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14. ABSTRACT The nucleolar tumor suppressor ARF plays an important role in the tumor surveillance of human cancer. We have found that ARF expression is absent from highly proliferative prostate adenocarcinomas and this correlates with the increased expression of the p53-independent target of NPM. We have created and characterized an immortalized ARF-null Prostate Epithelial cell line. In addition we have shown that alterations in NPM levels can have dramatic effects on the androgen-dependent cancer cell line, LNCaP, but PC3 cells which are androgen-independent. We have previously shown that ARF inhibits NPM's nuclear export and cell cycle progression in a p53-independent manner. Under this proposal we have defined the mechanism by which this happens. Specifically, ARF binds to NPM preventing its ability to carry both small and large ribosomal subunits out of the nucleus. This results in a decrease in protein synthesis and growth rates contributing to ARF's tumor suppressor function. We are beginning to further define the role of ARF in prostate cell growth. These studies are opening the door to new therapeutic targets in prostate cancer; namely protein synthesis.					
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## INTRODUCTION

Prostate cancer is the second leading cause of cancer related deaths in the US male population (1). With the development of prostate specific antigen (PSA) screening the number of prostate cancer cases identified has risen, and the number of deaths has begun to decrease. However, the molecular mechanisms underlying the development and progression of prostate

	p53	p21	Mdm2	ARF	NPM
-	60 (97%)	65 (100%)	32 (53%)	51 (96%)	35 (56%)
+	2 (3%)	0 (0%)	28 (47%)	2 (4%)	28 (44%)

**Table 1. Protein Expression in Prostate Adenocarcinomas**  
Human prostate tissue samples were analyzed by immunohistochemistry for the indicated proteins. Proteins were scored for their expression above (+) or at/below (-) wild type levels. The number of samples scored positive or negative is indicated with the percentage of the positive or negative in parentheses.

cancer are still largely unknown. The most mutated gene associated with human cancer in general, the p53 tumor suppressor, has been reported to play a role in the development and progression of prostate cancer (2, 3). p53 is regulated by the ARF tumor suppressor which imparts its control over cell proliferation though

both p53-dependent and -independent mechanisms, with the former involving the nucleolar sequestration of the p53 negative regulator, Mdm2 and the latter only recently discovered by our laboratory (4, 5). While tumors of epithelial cell origin are rare in *Arf*<sup>-/-</sup> or *p53*-null mice, animals lacking both *Arf* and *p53* display numerous tumors of this type demonstrating a role for ARF in preventing epithelial cell tumorigenesis (6-9). In addition, introduction of ARF into *p53*<sup>-/-</sup> MEFs results in cell cycle arrest (9). In numerous human cancers, the frequency of loss of the *Arf* tumor suppressor is second only to mutation of p53, providing critical evidence of *Arf*'s role in preventing tumorigenesis throughout the body, irregardless of cell type. However, the result of loss of ARF function in the prostate is unknown. The preliminary data for my original proposal indicated that prostate adenocarcinomas typically maintain wild type p53 (97%), but lose ARF (96%) expression. These data suggest ARF loss may play a role in the development and/or progression of prostate cancer. The goal of this study is to understand the role of the *Arf* tumor suppressor in prostate cell growth. We hypothesize that ARF utilizes p53-dependent and -independent mechanisms to regulate prostate cell proliferation.

## BODY

### Creation of an *Arf*<sup>-/-</sup> Prostate Epithelial Cell Line

To begin to address the role of ARF in PEpC growth, prostates were isolated from 8 week old *Arf*<sup>+/+</sup> and *Arf*<sup>-/-</sup> mice. The organs were diced into pieces approximately 1mm<sup>3</sup> in size and plated in defined media to select for prostate epithelial cell growth. As shown in Figure 1, cells cultures were grown from isolated prostates. Cells from the *Arf*<sup>-/-</sup> prostates grew well, but the wild-type cells were unable to proliferate beyond the 2<sup>nd</sup> passage. The *Arf*<sup>-/-</sup> prostate cells were then subjected to a 3T3 protocol in which 3x10<sup>5</sup> cells were passaged every 3 days to demonstrate their proliferative capacity. As shown in Figure 2A, the *Arf*<sup>-/-</sup> prostate cells were

able to be passaged 15 times without inhibition in growth indicating they are immortal. In fact the *Arf*<sup>-/-</sup> prostate cells have been passaged over 40 times without a decrease in growth (data not shown). In addition, an *Arf*<sup>-/-</sup> prostate cell growth curve shows that the cells can grow to confluency in a 10 cm dish (Figure 2B). Finally, *Arf*<sup>-/-</sup> prostate cells are not transformed as they cannot form colonies in soft agar (Figure 2C). Taken together these data indicate that the cell line created from *Arf*<sup>-/-</sup> prostates are immortal but not transformed.

To verify that the cultured cells were in fact epithelial in origin, *Arf*<sup>-/-</sup> prostate cells were seeded onto glass cover slips in defined media and incubated overnight. The cells were fixed and stained with antibodies against prostate epithelial (anti-CD38) and stromal (anti-CD54) markers (10). As shown in Figure 3, the *Arf*<sup>-/-</sup> prostate cells stained strongly for CD-38, the prostate epithelial marker, but showed almost no CD-54 signal indicating the cells were epithelial in origin not stromal.

After passaging the *Arf*<sup>-/-</sup> PEpC several times, we next determined if they maintained genomic integrity. *Arf*<sup>-/-</sup> PEpC (passage 20) were fixed and DNA was stained with propidium iodide (PI) and DNA content was analyzed by flow cytometry. As shown in Figure 4A, the *Arf*<sup>-/-</sup> PEpC contain 2N and 4N DNA with little to no >4N cells indicating these cells can maintain a proper DNA content. In addition, late passage *Arf*<sup>-/-</sup> PEpC were subjected to a colcemid block and metaphase spread analysis to determine chromosome number. As shown in Figure 4B, *Arf*<sup>-/-</sup> PEpC maintain normal chromosome number over several passages. Taken together these data demonstrate that *Arf*<sup>-/-</sup> PEpC remain diploid.

Since *Arf*<sup>-/-</sup> PEpC remain diploid as shown in Figure 4, this indicates that while the cells have lost *Arf*, p53 function remains intact. To verify this, *Arf*<sup>-/-</sup> PEpC were treated with etoposide to induce DNA strand breaks which will activate a p53 response. As shown in Figure 5A, treatment with 50 mM etoposide for 18 h results in cell cycle arrest. To show this is a p53-dependent cell cycle arrest the levels of p21, a downstream target of p53, were determined by western blot. As shown in Figure 5B, p21 was induced in response to etoposide treatment indicating that *Arf*<sup>-/-</sup> PEpC have an intact p53 response. In addition to having an intact p53 response to DNA damage, ARF expression in *Arf*<sup>-/-</sup> PEpC should also result in cell cycle arrest. As shown in Figure 6, overexpression of p19ARF in *Arf*<sup>-/-</sup> PEpC results in the inhibition of cell cycle progression indicating that this cell line has an intact ARF response.

In addition to expression of prostate epithelial markers, PEpC require androgen signaling for survival (11). As shown in Figure 7E, the *Arf*<sup>-/-</sup> PEpC express the androgen receptor by western blot analysis. Using an androgen receptor antagonist, bicalutamide from AstraZeneca, I was able to demonstrate that *Arf*<sup>-/-</sup> PEpC do not proliferate well (Figure 7A and B) as shown by a growth curve. In addition, treatment of *Arf*<sup>-/-</sup> PEpC with bicalutamide for 48 h blocks BrdU incorporation into replicating DNA (Figure 7C). Taken together these data indicate that *Arf*<sup>-/-</sup> PEpC express the androgen receptor and require androgen signaling for growth.

Previous studies have shown that Androgen Receptor signaling is required for phosphorylation of NPM, a p53-independent target of the ARF nucleolar tumor suppressor (12, 13). As shown in Figure 7D, inhibition of androgen receptor signaling in *Arf*<sup>-/-</sup> PEpC with bicalutamide results for 96 h results in a loss in phosphorylation of NPM at Thr198 (top panel).

This correlates with a decrease in total NPM levels in these cells (Figure 7B bottom panel). Interestingly, the androgen independent prostate tumor cell line PC3 has a detectable level of NPM (data not shown) indicating that androgen independence may result from the ability to stabilize NPM in the absence of androgen receptor activity.

**Nucleophosmin (NPM/B23) is a p53-independent target of nucleolar ARF tumor suppression.**

Our lab and others have previously shown that the nucleolar tumor suppressor, ARF, can bind to NPM (5). We have also shown that ARF inhibits NPM nuclear/cytoplasmic trafficking which correlates with an inhibition of cell cycle progression in a p53-independent manner (5). However, the underlying mechanism of how ARF-induced inhibition of NPM nuclear export regulates cell cycle progression remained undiscovered.

NPM is a key regulator in the regulation of a number of processes including centrosome duplication, maintenance of genomic integrity, and ribosome biogenesis. While the mechanisms underlying NPM function are largely uncharacterized, NPM loss results in severe dysregulation of developmental and growth-related events. We determined that NPM utilizes a conserved CRM1-dependent nuclear export sequence in its amino terminus to enable its shuttling between the nucleolus/nucleus and cytoplasm. In search of NPM trafficking targets, we biochemically purified NPM-bound protein complexes from HeLa cell lysates. Consistent with NPM's proposed role in ribosome biogenesis, we isolated ribosomal protein L5 (rpL5), a known chaperone for the 5S rRNA. Direct interaction of NPM with rpL5 mediated the colocalization of NPM with maturing nuclear 60S ribosomal subunits, as well as newly exported and assembled 80S ribosomes and polysomes. Inhibition of NPM shuttling or loss of *NPM* blocked the nuclear export of rpL5 and 5S rRNA, resulting in cell cycle arrest and demonstrating that NPM and its nuclear export provide a unique and necessary chaperoning activity to rpL5/5S (SEE ATTACHED PAPER #1 IN APPENDICES, "Nucleophosmin is Essential for Ribosomal Protein L5 Nuclear Export").

NPM is a critical target of ARF tumor suppression while, in the absence of *ARF*, NPM is capable of transforming cells. In addition to our preliminary findings that ARF is under expressed in 96% of prostate adenocarcinomas, more detailed analysis of NPM levels show that over 82% of proliferating (Ki-67 positive) prostate adenocarcinomas overexpress NPM with 44% grossly overexpressing the gene. We have reported the mechanism for NPM's regulation of cell growth; NPM directs the nuclear export of both 40S and 60S ribosomal subunits. Transduction of NPM shuttling-defective mutants or loss of *Npm*, inhibited nuclear export of both the 40S and 60S ribosomal subunits, reduced the available pool of cytoplasmic polysomes, and diminished overall protein synthesis without affecting ribosomal RNA processing. Modest increases in NPM expression amplified the export of newly synthesized ribosomal RNAs, resulting in increased rates of protein synthesis and indicating that NPM is rate-limiting in this pathway. These results support the idea that NPM-regulated ribosome export is a fundamental process in growth and cellular transformation (SEE ATTACHED MANUSCRIPT #1 IN APPENDICES, "Nucleophosmin Directs Ribosome Nuclear Export and Cell Growth").

We next examined the effects of inhibition of NPM shuttling on S phase entry of two human prostate cancer cell lines, androgen-dependent LNCaP and androgen independent PC3. As shown in Figure 8, overexpression of NPM or the NPM shuttling mutant NPMdL did not alter BrdU incorporation into replicating DNA. However, the androgen-dependent cell line, LNCaP, showed a high sensitivity to inhibition of NPM shuttling (>5 fold decrease, Figure 9B). Interestingly, the level of wild type NPM overexpression also affected BrdU incorporation. As shown in Figure 9A (arrows), high levels of NPM expression resulted in an inhibition of BrdU incorporation into replicating DNA (>5 fold Figure 9B). However, lower levels of expression showed a slight increase in the number of BrdU positive cells (Figure 9B). Taken together with the data from the *Arf*<sup>-/-</sup> PEpC which show sensitivity of NPM levels to androgen signaling (figure 7D), and the data showing high NPM levels in prostate adenocarcinomas (SEE ATTACHED MANUSCRIPT #1 IN APPENDICES, “Nucleophosmin Directs Ribosome Nuclear Export and Cell Growth”) this data indicates that targeting protein synthesis could be a useful therapeutic target for the treatment of prostate cancer.

To examine the effects of *Arf* loss on PEpC protein synthesis rate, wild-type and *Arf*<sup>-/-</sup> PEpC were labeled with <sup>35</sup>S-Methionine for the indicated times and total trichloroacetic acid-precipitable proteins were counted for label incorporation. As shown in figure 10, *Arf*<sup>-/-</sup> PEpC produce 4-fold more protein over a 24 time course than an equal number of wild type PEpC. These data indicate that *Arf* is required to regulate protein synthesis in PEpC.

Our previous studies have shown that NPM can transform immortalized *Arf*<sup>-/-</sup> MEFs. While loss of *Arf* results in an increase in protein synthesis rates of PEpC, overexpression of NPM cannot increase growth rates as determined by foci assay (Figure 11A), nor transform them as determined by colony formation in soft agar (Figure 11B). In addition, classical transforming genes such as a constitutively active *RasV12* and *Myc* alone and in combination cannot increase growth rates of (Figure 11A) or transform (Figure 11B) *Arf*<sup>-/-</sup> PEpC. This is not unprecedented as bone marrow derived cells isolated from *Arf*<sup>-/-</sup> mice could not be transformed with *RasV12* or *Myc* alone (14). However, in combination *RasV12* and *Myc* overexpression were able to transform *Arf*<sup>-/-</sup> bone marrow derived cells. While these findings are discouraging, there are still several avenues to determining the requirements for transformation of *Arf*<sup>-/-</sup> PEpC such as the system used by Hahn and colleagues (15) utilizing several factors to transform cells.

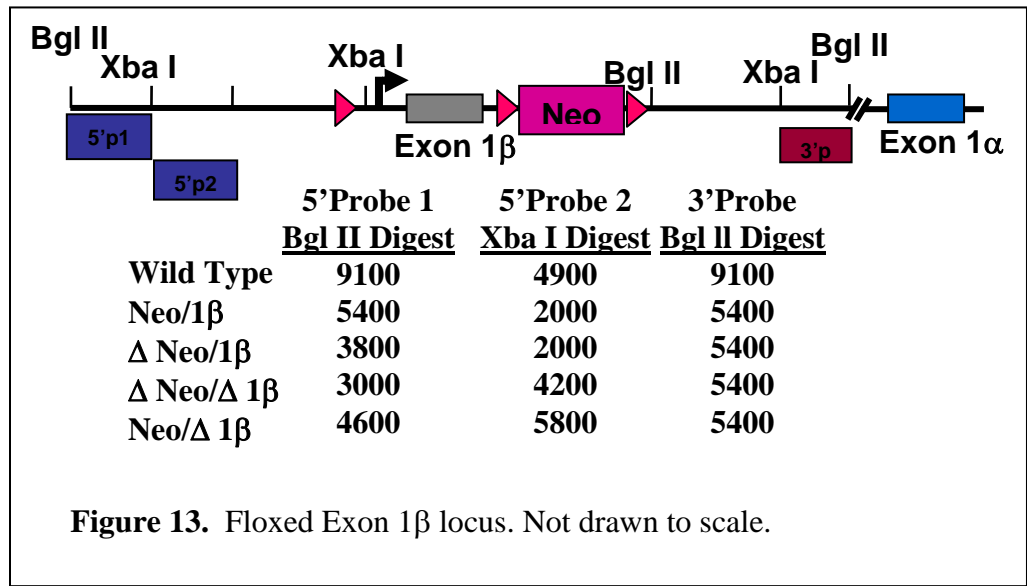
To further support these findings we have performed Electron Microscopy on Mouse Embryo Fibroblasts (MEF) isolated from *Npm*<sup>+/+</sup> and *Npm* hypomorphic (*Npm*<sup>h/h</sup>) mice. As shown in Figure 12, loss of *Npm* results in a thickening of the nuclear membrane (arrows) indicating a buildup of ribosomal subunits that cannot exist the nucleus (upper panels). In the cytoplasm of *Npm*<sup>h/h</sup> MEF there is also a paucity of rough ER compared to wild-type (diamond headed arrows, upper panels). Finally, the morphology of the nucleolus is markedly altered with a large increase in vacant areas in *Npm*<sup>h/h</sup> nucleoli versus *Npm*<sup>+/+</sup> nucleoli (bottom panels).

Taken together these data provide evidence that ARF can regulate PEpC growth and proliferation through its interaction with NPM. The effects on the other ARF-interacting proteins will be explored, but as discussed below, a few minor difficulties will need to be dealt with first.

**Difficulties and actions taken**

Due to the difficulty in transforming *Arf*<sup>-/-</sup> PEPc, I have not been able to assess the response of the ARF-interacting genes, *Mdm2*, *Bop1*, and *Npm* to true transforming signals in the prostate. I am in the process of assessing their mRNA and protein levels in response to RasV12, Myc and the combination of the two which are classical transforming signals in rodent cells. Assessing the response of these genes to classically transforming signals will offer insights into why *Arf*<sup>-/-</sup> PEPc are not transformed. In addition, I have finally received a few samples from the Tissue Procurement Core at Washington University which I will use to asses the levels of ARF, NPM, Mdm2, and BOP1 at the mRNA level. These studies will also offer more insight into the role of these genes in prostate cancer.

I have also run into difficulty cloning the targeting vector for creation of the conditional *Arf* knockout mouse (see figure 13). I have been able to clone both homology arms individually



into the targeting vector. However, when all attempts to get the 2<sup>nd</sup> homology arm, whether it be the 5' or 3' homology arm have failed. The CpG island just 5' to Arf exon 1β is causing problems and

results in the deletion of a portion of the 5'HA around exon 1β. Four members of the lab including me have attempted the cloning reaction from beginning to end. I have attempted using several different bacterial cell lines (DH5a, JM109, XL10 Gold, ABLE C, and SCS110) some of which are defective in recombination genes or are purported to tolerate “toxic” plasmids. I am in the midst of attempting to clone into two new targeting vectors as well as recreating the targeting strategy. While doing this, I have begun a study to look at the effects of ARF loss on a conditional knockout model of prostate cancer. Work performed by Dr Henry Sucov’s lab (16, 17) shows that selective deletion of the RXRα results in PIN lesions but does not result in metastatic prostate cancer. I have received these RXRα floxed mice from Dr Sucov as well as the Pb-Cre mice from the NCI. I am breeding the Pb-Cre mice onto the *Arf*<sup>-/-</sup> background and will then cross the Pb-Cre/*Arf*<sup>-/-</sup> mice to the RXRα floxed mice to determine the effects of *Arf* loss on the progression and extent of disease in the mice.



## KEY RESEARCH ACCOMPLISHMENTS

- Isolated and established wild-type and *Arf*<sup>-/-</sup> prostate epithelial cell lines.
- Wild-type prostate epithelial cells have limited proliferation in culture.
- *Arf*<sup>-/-</sup> prostate epithelial cells are immortal in culture, but not transformed.
- *Arf*<sup>-/-</sup> prostate epithelial cells maintain ploidy as well as an intact p53 response in culture.
- *Arf*<sup>-/-</sup> prostate epithelial cells make 4-fold more protein over a 24 h time course than wild-type cells.
- NPM is a potent transforming oncogene in the absence of *Arf* in Mouse Embryo Fibroblasts but not in Prostate Epithelial Cells.
- NPM is overexpressed in 82% and highly overexpressed in 44% of proliferating prostate adenocarcinomas.
- “Nucleophosmin is Essential for Ribosomal Protein L5 Nuclear Export”, Yue *et al. Molecular and Cellular Biology* (2006) **26**:3798-3809.
- “Nucleophosmin Directs Ribosome Nuclear Export and Cell Growth”, Maggi *et al. Submitted* (2006).

## REPORTABLE OUTCOMES

- “Nucleophosmin is Essential for Ribosomal Protein L5 Nuclear Export”, Yue *et al. Molecular and Cellular Biology* (2006) **26**:3798-2809.
- “Nucleophosmin Directs Ribosome Nuclear Export and Cell Growth”, Maggi *et al. Submitted* (2006).
- “What is the role of the ARF tumor suppressor in Prostate Tumorigenesis?” Oral Presentation at the Washington University Signaling/Cell Cycle Group (December 2004).
- “Regulation of Growth and Transformation by Nucleophosmin-mediated Ribosome Export” Invited Oral Presentation at the 2<sup>nd</sup> Annual Washington University Postdoc Symposium (February 2006).
- “Nucleophosmin Directs Ribosome Nuclear Export and Cell Growth” Co-Winner of Best Poster at the 1<sup>st</sup> Annual Washington University Signaling/Cell Cycle Retreat (April 2006).
- Creation and characterization of an *Arf*<sup>-/-</sup> prostate epithelial cell line.

## CONCLUSIONS

This proposal was designed to investigate the role of the nucleolar tumor suppressor ARF in the development of prostate cancer. In the first 18 months of support I have generated a substantial amount of data which is laying the foundation for future studies. I have isolated and characterized an immortal prostate epithelial cell line. In addition, I have defined a novel and very important mechanism for ARF-induced cell cycle arrest that is independent of p53. The role of NPM and protein synthesis in prostate cancer is an exciting and emerging field of study which has also defined a pathway with promising therapeutic targets for prostate cancer

treatment. I have begun work on all three aims and am looking forward to a very productive 6 months to finish the work outlined in this proposal.

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## APPENDICES

1. “Nucleophosmin is Essential for Ribosomal Protein L5 Nuclear Export”, Yue *et al.* *Molecular and Cellular Biology* (2006) **26**:3798-2809.
2. “Nucleophosmin Directs Ribosome Nuclear Export and Cell Growth”, Maggi *et al.* Submitted (2006).
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## SUPPORTING DATA

**Figure 1.** *Arf*<sup>-/-</sup> Prostate Cell Cultures.

**Figure 2.** *Arf*<sup>-/-</sup> Prostate Cells are Immortal not Transformed.

**Figure 3.** *Arf*<sup>-/-</sup> Prostate Cells are Epithelial Cells.

**Figure 4.** *Arf*<sup>-/-</sup> PEpC are Diploid.

**Figure 5.** *Arf*<sup>-/-</sup> PEpC Have an Intact p53 Response.

**Figure 6.** p19ARF Overexpression Inhibits *Arf*<sup>-/-</sup> PEpC Cell Cycle Progression.

**Figure 7.** Androgen Receptor Signaling is Required for *Arf*<sup>-/-</sup> PEpC Growth.

**Figure 8.** PC3 Cell S Phase Progression Is Not Affected By NPM Shuttling.

**Figure 9.** LNCaP Cell S Phase Progression Is Affected By Inhibition of NPM Shuttling or High Expression of NPM.

**Figure 10.** *Arf* loss Increases Protein Synthesis Rates of PEpC.

**Figure 11.** *Arf*<sup>-/-</sup> PEpC Cannot Be Transformed.

**Figure 12.** Loss of *Npm* Inhibits Ribosome Nuclear Export and Alters Nucleolar Morphology.

# Nucleophosmin Is Essential for Ribosomal Protein L5 Nuclear Export

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**Nucleophosmin (NPM/B23) is a key regulator in the regulation of a number of processes including centrosome duplication, maintenance of genomic integrity, and ribosome biogenesis. While the mechanisms underlying NPM function are largely uncharacterized, NPM loss results in severe dysregulation of developmental and growth-related events. We show that NPM utilizes a conserved CRM1-dependent nuclear export sequence in its amino terminus to enable its shuttling between the nucleolus/nucleus and cytoplasm. In search of NPM trafficking targets, we biochemically purified NPM-bound protein complexes from HeLa cell lysates. Consistent with NPM's proposed role in ribosome biogenesis, we isolated ribosomal protein L5 (rpL5), a known chaperone for the 5S rRNA. Direct interaction of NPM with rpL5 mediated the colocalization of NPM with maturing nuclear 60S ribosomal subunits, as well as newly exported and assembled 80S ribosomes and polysomes. Inhibition of NPM shuttling or loss of NPM blocked the nuclear export of rpL5 and 5S rRNA, resulting in cell cycle arrest and demonstrating that NPM and its nuclear export provide a unique and necessary chaperoning activity to rpL5/5S.**

As the most prominent of subnuclear structures, the nucleolus has long been recognized as the site of active transcription of rRNA and ribosome assembly (8). Various nucleolar proteins, RNAs, and other factors have been implicated in the complex process of ribosome production and maturation (18). Recently, several groups reported the successful isolation and mapping of the mammalian nucleolar proteome (1, 2, 44). While these studies clearly identified proteins and ribonucleoproteins with purported roles in ribosome biogenesis, a surprising number of proteins within the nucleolar proteome (>100) have no known function. In previous decades, it was assumed that all nucleolar proteins must somehow contribute to static ribosome biogenesis simply by virtue of their localization. However, more-recent findings have demonstrated that the nucleolus is a dynamic subnuclear organelle which regulates numerous cellular processes, prompting a broadened view of the potential functions of nucleolar proteins (28).

Nucleophosmin (NPM/B23) is an abundant phosphoprotein that resides within the granular regions of the nucleolus (46). Proliferating cells express NPM at high levels (9, 13), and NPM has been associated with a variety of cellular events, including ribosomal biogenesis, protein chaperoning, and centrosome duplication (13, 23, 35, 36). Structurally, NPM is present in both monomeric and multimeric states, although NPM multimers appear predominant in the nucleolus and may be crucial for the assembly of maturing ribosomes (33, 34, 53). Furthermore, NPM, along with other nucleolar proteins, is believed (or has been shown) to actively mobilize into distinct subcellular pools, supporting the notion that NPM trafficking may be

essential for its (proper) function (6). Indeed, NPM's transit from the nucleolus/nucleus is an essential event in S phase progression; when NPM export was inhibited by the nucleolar tumor suppressor ARF, cells arrested in G<sub>1</sub> (7). Moreover, loss of NPM expression results in severe attenuation of cellular proliferation and increased apoptosis (5, 7, 16, 19), underscoring NPM's indispensable role within the cell.

Given that nuclear export of NPM promotes cell growth, we aimed to further elucidate the crucial roles of NPM's trafficking. While numerous proteins, such as Mdm2, cdc14p, and telomerase reverse transcriptase, are topologically restrained in the nucleolus following receipt of defined cellular cues, newly synthesized ribosomal subunits must be exported from the nucleolus to promote proper protein translation in the cytosol (45). Recent work with *Xenopus laevis* and *Saccharomyces cerevisiae* has shown that nuclear export of ribosomes utilizes the CRM1-RanGTP export receptor pathway (20) as well as the conserved nuclear adaptor protein NMD3 (51). While investigating the critical nature of NPM trafficking, we noted that NPM's exit from the nucleus also involved the classical CRM1-dependent nuclear export pathway. In search of proteins that are targeted for NPM-mediated nuclear export, we observed that nuclear and cytosolic NPM proteins directly bound to the ribosomal L5 protein (rpL5), a 60S subunit protein that chaperones the 5S rRNA into the nucleolus and out into the cytosol (31). Here we report that NPM mediates rpL5/5S nuclear export through a CRM1-dependent mechanism, allowing NPM to directly access the maturing ribosome and potentially regulate the protein translational machinery.

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## MATERIALS AND METHODS

**Cell culture.** HeLa and NIH 3T3 cells and wild-type (WT) mouse embryonic fibroblasts (MEFs) (ArtisOptimus, Carlsbad, CA) were maintained in Dulbecco's

modified Eagle's medium with 10% fetal bovine serum, 2 mM glutamine, 0.1 mM nonessential amino acids, and 100 U penicillin and streptomycin.

**Plasmid constructs.** Vectors encoding full-length His-tagged murine NPM are described elsewhere (7). The His epitope-tagged NPM coding sequence was subcloned into pcDNA3.1 (Invitrogen) and pEGFP (Clontech) vectors. His-NPM<sub>Δ42-61</sub>, His-NPM<sub>Δ62-83</sub>, or His-NPMΔL mutants were generated using the primers 5'-GAAATGAGCACCAGGCAGCAAGCAATGAAC-3' (sense) and 5'-GTTTCATTGCTTCTGCTGGTGCTCATTTTC-3' (antisense), 5'-GTTACACATCGTAGAGCAACCAACAGTTTCC-3' (sense) and 5'-GGAACTGTTGGTTGCTCTACGATGTGTAAAC-3' (antisense), or 5'-GAAATGAGCACCAGGCGTCAGCAAGAACGGTC-3' (sense) and 5'-CTAACTGACCGTCTTCTGCTACGCCTGGTGCTCATTTTC-3' (antisense), respectively, by QuikChange mutagenesis (Stratagene). A myc-tagged NPC-M9 (40) in pcDNA3 and a green fluorescent protein (GFP)-tagged rpL5 plasmid (41) were generous gifts from Alan Diehl (University of Pennsylvania) and Joachim Hauber (Universitat Erlangen-Nürnberg).

**Heterokaryon assay.** HeLa cells ( $2 \times 10^5$ ) were seeded onto glass coverslips and transfected with plasmids. NIH 3T3 cells ( $6 \times 10^5$ ) were seeded onto the HeLa cells 24 h posttransfection. Cocultures were then incubated for 30 min with cycloheximide (100 μg/ml), followed by incubation with 50% polyethylene glycol in phosphate-buffered saline for 105 s. Cocultures were incubated with Dulbecco's modified Eagle's medium containing cycloheximide (100 μg/ml) for an additional 4 h. Heterokaryons were fixed and stained with a rabbit anti-His antibody (Santa Cruz) or mouse anti-myc antibody (Zymed), followed by either fluorescein isothiocyanate-conjugated or rhodamine X-conjugated anti-rabbit or anti-mouse immunoglobulin (Pierce) as described previously (7). Nuclei were stained with Hoechst (Sigma). Fluorescent signals were detected using a Nikon epifluorescence compound microscope ( $\times 100$ ) fitted with a Nikon FDX-35 camera.

**Immunoprecipitation and Western blot analysis.** Cells were transfected as recommended by the manufacturer (Amaya) with vectors encoding His-NPM, His-NPMΔL, and GFP-rpL5 and lysed in binding buffer (25 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40) 48 h after the Nucleofector process. Primary antibody to the NPM N terminus (custom rabbit; Sigma Genosys), GFP (Santa Cruz), His (Santa Cruz), rpL5 (12), or nonimmune rabbit serum (NRS) was added to the binding reaction mixtures. Immune complexes were precipitated with protein A-Sepharose (Amersham). The precipitated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. NPM, His-tagged proteins, and GFP-tagged proteins were visualized by direct immunoblotting with NPM (Zymed), His (Santa Cruz), and rpL5 and GFP (Santa Cruz) antibodies, respectively.

**Fluid phase liquid chromatography.** For affinity chromatography, a rabbit polyclonal antibody recognizing the N terminus of NPM (Sigma) was coupled to *N*-hydroxysuccinimide-activated Sepharose (Amersham). HeLa cells were lysed in 20 mM Tris, pH 7.4, and 0.1% Tween 20 and sonicated. Lysates (600 μg) were injected onto the NPM affinity column, washed with 20 mM Tris, and eluted with an increasing NaCl gradient (0.1 to 1 M) using BioLogic fluid phase liquid affinity chromatography and HR software (Bio-Rad). Fractions were precipitated with trichloroacetic acid (TCA). Proteins were resuspended in 1 M Tris-HCl (pH 7.4), separated by SDS-PAGE, and visualized with Coomassie blue stain (Sigma).

**Proteomic analysis.** Proteins from fluid phase liquid affinity chromatography fractions were precipitated with TCA and resuspended in Laemmli buffer. SDS-PAGE-separated proteins were stained with SYPRO-Ruby (Bio-Rad). Bands of interest were excised and processed for trypsin digestion. Tryptic peptides were calibrated with Sequazyme peptide mass standard kit (PE Biosystems) and analyzed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (Voyager DE Pro; Applied Biosystems). Identification of proteins was performed using MS-Fit software (<http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm>). MALDI-TOF spectra and sequences were verified using a 4700 Proteomics tandem mass spectrometry system (Applied Biosystems). Identified proteins were additionally verified by direct Western blot analysis.

**Bacterial protein purification.** BL21 cells were transformed with pET28a vectors encoding NPM, NPMΔL, rpL5, and p27kip1 proteins. Protein production was induced for 3 h with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Harvested cells were lysed in phosphate-buffered saline containing protease inhibitors and 1% Triton X-100 with sonication. Cleared lysates were subjected to affinity purification using Ni-nitrilotriacetic acid columns as described by the manufacturer (Sigma). Purified proteins were separated by SDS-PAGE and visualized for purity using Coomassie blue stain.

**Subcellular fractionation.** HeLa cells were subjected to the Nucleofector process with scrambled or small interfering NPM RNAs or control vector, His-NPM, and His-NPMΔL and harvested. Pellets containing equal cell numbers were resuspended in HEPES buffer (10 mM HEPES, pH 7.4, with 4 mM MgCl<sub>2</sub>, 1 mM

phenylmethylsulfonyl fluoride [PMSF], 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin) and lysed with a syringe. Lysates were pelleted, and the supernatant was saved as the cytoplasmic fraction. The pellet was resuspended in fractionation buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 4 mM MgCl<sub>2</sub>, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin), subjected to Dounce homogenization, layered over a cushion of sucrose (45%, wt/vol, in fractionation buffer), and centrifuged. The pellet was washed and resuspended in EBC buffer (50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin, 1 mM NaF, 10 mM NaVO<sub>4</sub>, β-glycerophosphate). Nuclear or cytoplasmic protein was subjected to SDS-PAGE. Superoxide dismutase (SOD; Cu/Zn-specific form), lamin A/C, and rpL5 proteins were visualized by direct immunoblotting with anti-SOD (Calbiochem), anti-lamin A/C (Santa Cruz), and anti-rpL5 antibodies (12), respectively. Similarly, total RNA was isolated from the fractions obtained above and separated on formaldehyde-agarose gels. Separated RNA from each nuclear and cytoplasmic fraction was analyzed by Northern blotting using a probe specific for the 5S rRNA. The 5S rRNA probe was obtained by PCR using HeLa cell genomic DNA as the template and the following primers: sense, 5'-CCTTCACGCTCTACGCCATACC-3'; antisense, 5'-GCCAAGAAAAGCCTACAGCAGG-3'. The PCR product was cloned and confirmed by sequencing.

**RNA FISH.** HeLa cells were subjected to the Nucleofector process with pcDNA3.1 His, His-tagged NPM, or His-NPMΔL and plated on coverslips. Cells were subjected to RNA fluorescence in situ hybridization (FISH) as described previously (3) using a tetramethyl rhodamine isocyanate (TRITC)-labeled 5S rRNA probe (Genedetec). DNA was counterstained with DAPI (4',6'-diamidino-2-phenylindole).

**Ribosome fractionation.** Cells were subjected to cytosolic and nuclear ribosome fractionation, and lysates were separated on sucrose gradients as previously described (48). RNA was continuously monitored over the gradient by measuring UV absorbance at 254 nm. Fractions were collected, and proteins were precipitated with TCA. Proteins were separated by SDS-PAGE and immunoblotted with antibodies recognizing NPM (Zymed) and rpL5.

## RESULTS

**NPM nuclear export requires a CRM1-dependent nuclear export signal involving leucines 42 and 44.** NPM is a ubiquitously expressed nucleolar phosphoprotein capable of regulated nuclear import (6). When NPM is transiently expressed in mammalian cells, it localizes predominantly to the nucleolus. Moreover, using *in vivo* heterokaryon shuttling assays (50), we have previously shown that NPM readily shuttles between the nucleolus/nucleus and cytoplasm (7). NPC-M9, a nuclear hnRNP protein that readily mobilizes to the cytoplasm, serves as a shuttling control (40). To distinguish between human donor and murine acceptor nuclei, chromosomal DNA was stained with Hoechst, clearly demarcating greater heterochromatin foci of NIH 3T3 mouse cells (speckled pattern, Fig. 1, Hoechst). As shown in Fig. 1A, NPM readily shuttles out of the human nucleolus, into the fused cytoplasm, and back into the mouse acceptor nucleus/nucleolus.

Given that a wide range of shuttling proteins utilize the CRM1 transport protein for their nuclear export, we further investigated the underlying export mechanism of NPM both in the presence and absence of leptomycin B (LMB), a potent inhibitor of CRM1-mediated nuclear export (24). In the absence of LMB, NPM readily migrated from human nucleoli to mouse nucleoli (Fig. 1A). However, in the presence of LMB, NPM failed to shuttle and was restricted to human nucleoli within heterokaryons (92% inhibition; Fig. 1B). The addition of LMB did not hinder the nucleocytoplasmic trafficking of Myc-NPC-M9, an hnRNP that readily shuttles in a CRM1-independent nuclear export pathway (38).

A sequence alignment of NPM residues with known CRM1-dependent shuttling proteins revealed two motifs containing

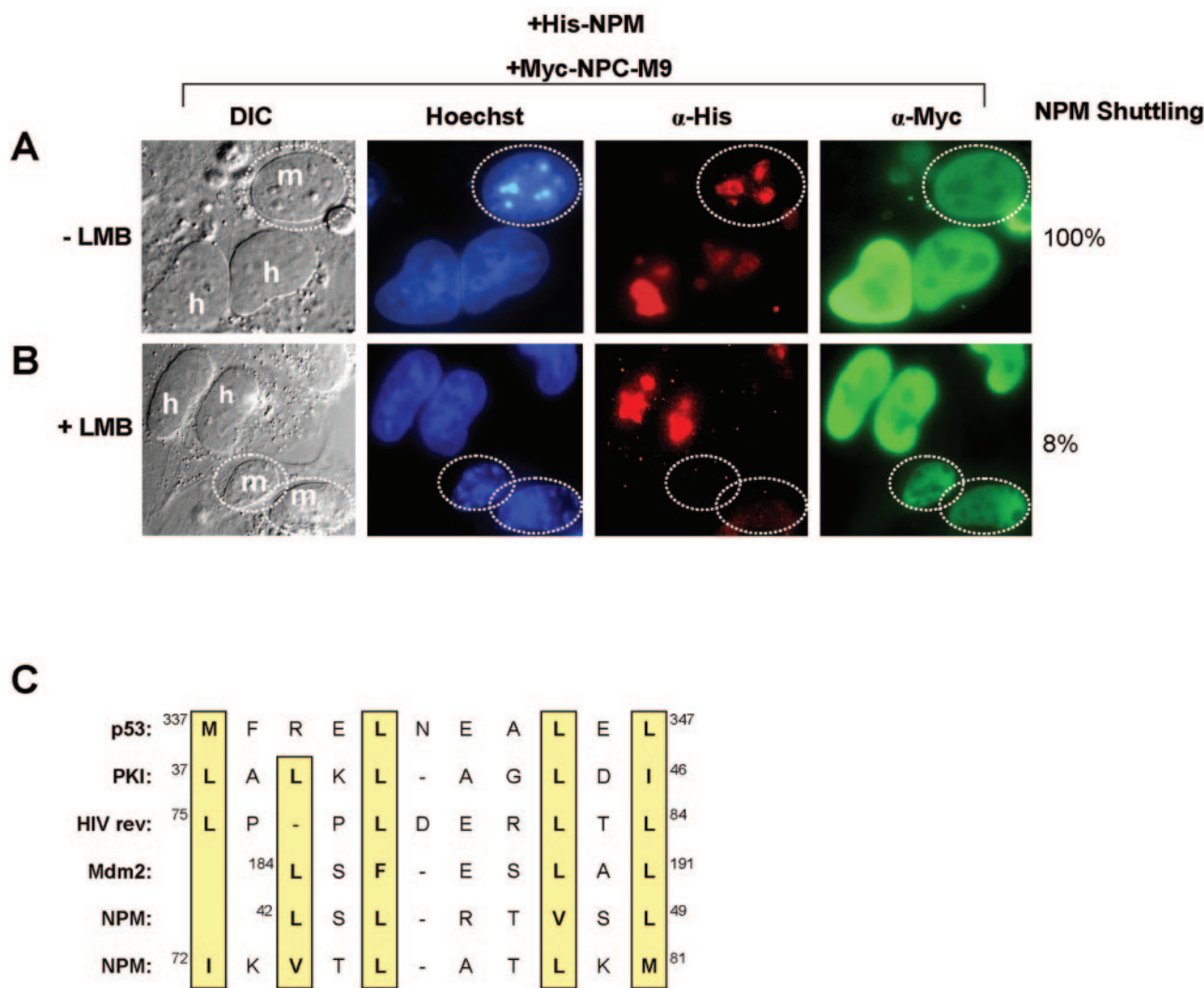


FIG. 1. Nuclear export of NPM is CRM1 dependent. NIH 3T3 cells were seeded onto HeLa cells that had been transfected with His-NPM in combination with Myc-NPC-M9 (shuttling control) in the (A) absence or (B) presence of LMB. Heterokaryons were incubated in media containing cycloheximide for an additional 4 h before fixation. Heterokaryon formation was verified by phase-contrast microscopy, while His-NPM and Myc-NPC-M9 proteins were visualized with antibodies against His (red) and Myc (green), respectively. DNA was stained with Hoechst. Mouse nuclei are demarcated with dotted circles. Human and mouse nuclei are labeled h and m, respectively. These data are representative of at least five independent heterokaryons formed for each transfection condition in three independent experiments. The percentages of His-NPM shuttling in heterokaryons are given. DIC, differential interference contrast;  $\alpha$ , anti. (C) Sequence alignment of putative NPM NESs with known NESs of CRM1-dependent nuclear export proteins (p53, protein kinase inhibitor [PKI], rev, and Mdm2). Critical hydrophobic residues are indicated in yellow.

short leucine-rich hydrophobic stretches of amino acids characteristic of CRM1-dependent nuclear export sequences (NESs) (Fig. 1C) (14, 15). In order to identify which region(s) of NPM contains its NES, we generated deletion mutants of NPM lacking either of the two potential NESs (NPM $_{\Delta 42-61}$  and NPM $_{\Delta 62-83}$ ). Using these NPM constructs, we again conducted interspecies heterokaryon assays. As shown in Fig. 2A, deletion of amino acids 42 to 61 of NPM (His-NPM $_{\Delta 42-61}$ ) prevented its shuttling (100% inhibition) to mouse nucleoli. Importantly, a myc-tagged NPC-M9 shuttling control readily shuttled in the same human-mouse heterokaryon, indicating that these heterokaryons were not impaired for nucleocytoplasmic shuttling in general. In contrast, deletion of amino acids 62 to 83 of

NPM (His-NPM $_{\Delta 62-83}$ ) did not prevent NPM from shuttling between human and mouse nucleoli (6% inhibition; Fig. 2B), revealing that the putative NES resides within amino acids 42 to 61 of the NPM protein.

Since the type of NES recognized and bound by the CRM1 export receptor is dependent on closely spaced hydrophobic amino acids (particularly leucines) (14, 15), we introduced point mutations into the corresponding leucine residues within the NES of NPM (Leu-42 and Leu-44 to Ala-42 and Ala-44). First, we tested this NPM mutant (designated NPMdL for double-leucine mutant) with Myc-NPC-M9 as a shuttling control. As expected, NPMdL was unable to transit from a human nucleus to the cytoplasm and into a murine nucleus (100%



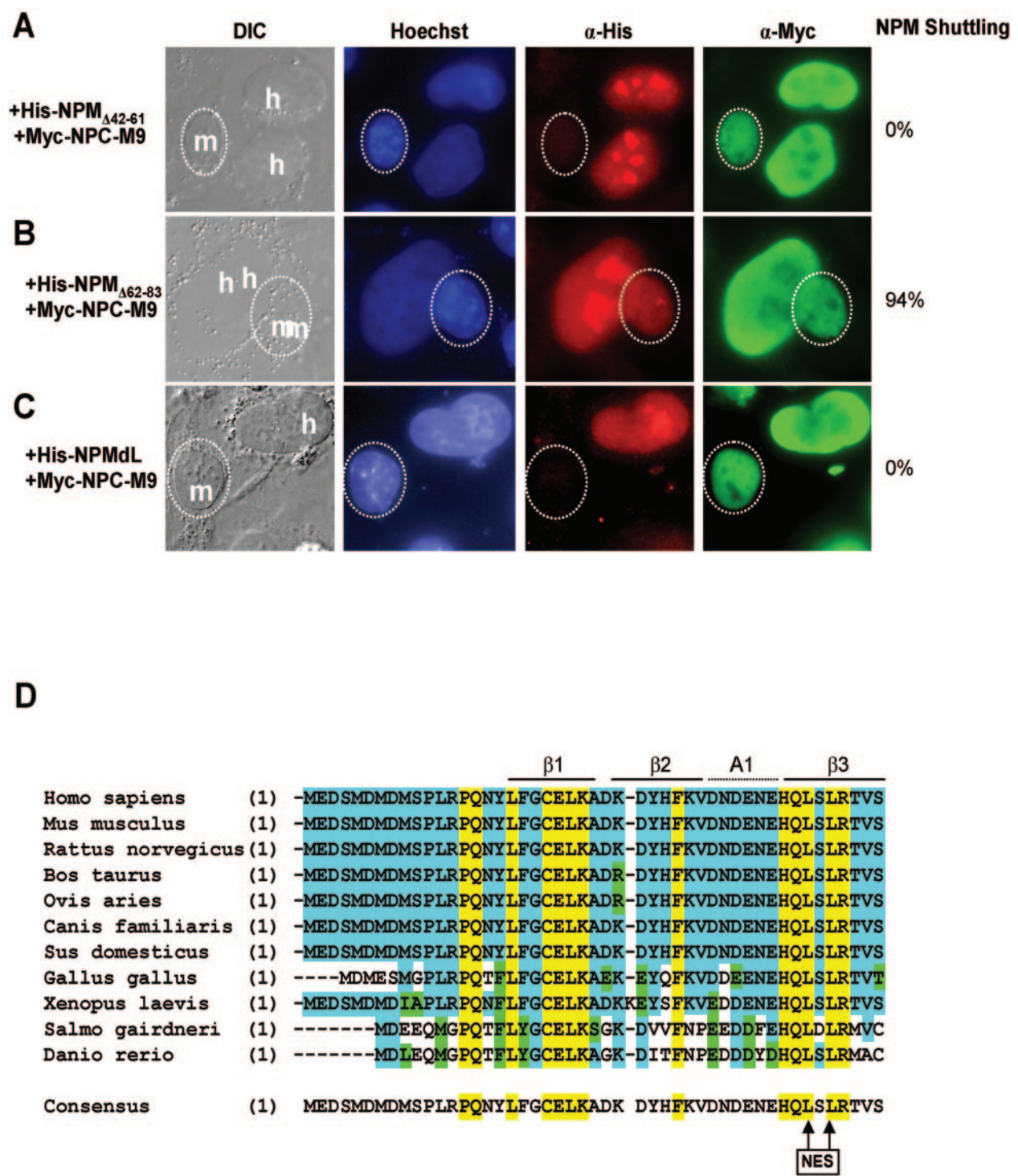


FIG. 2. Leucine 42 and leucine 44 are identified as critical nuclear export residues. NIH 3T3 cells were seeded onto HeLa cells that had been transfected with (A) His-NPM $_{\Delta 42-61}$ , (B) His-NPM $_{\Delta 62-83}$ , or (C) NPMdL in combination with Myc-NPC-M9. Ectopic NPM proteins and Myc-NPC-M9 proteins were visualized with antibodies against His (red) and Myc (green), respectively. DNA was stained with Hoechst. Mouse nuclei are demarcated with dotted circles. Human and mouse nuclei are labeled h and m, respectively. These data are representative of at least five independent heterokaryons formed for each transfection condition in three independent experiments. The percentages of His-NPM shuttling in heterokaryons are given. DIC, differential interference contrast;  $\alpha$ , anti. (D) Sequence alignment of NPM homologues throughout evolution. Identical residues in all species are marked yellow, identical residues in at least seven species are highlighted blue, and conserved residues are marked green. Crystal structure features are identified above the sequences. The consensus NPM sequence for all 11 identified homologues is given, with conserved nuclear export leucines 42 and 44 marked with arrows (NES).

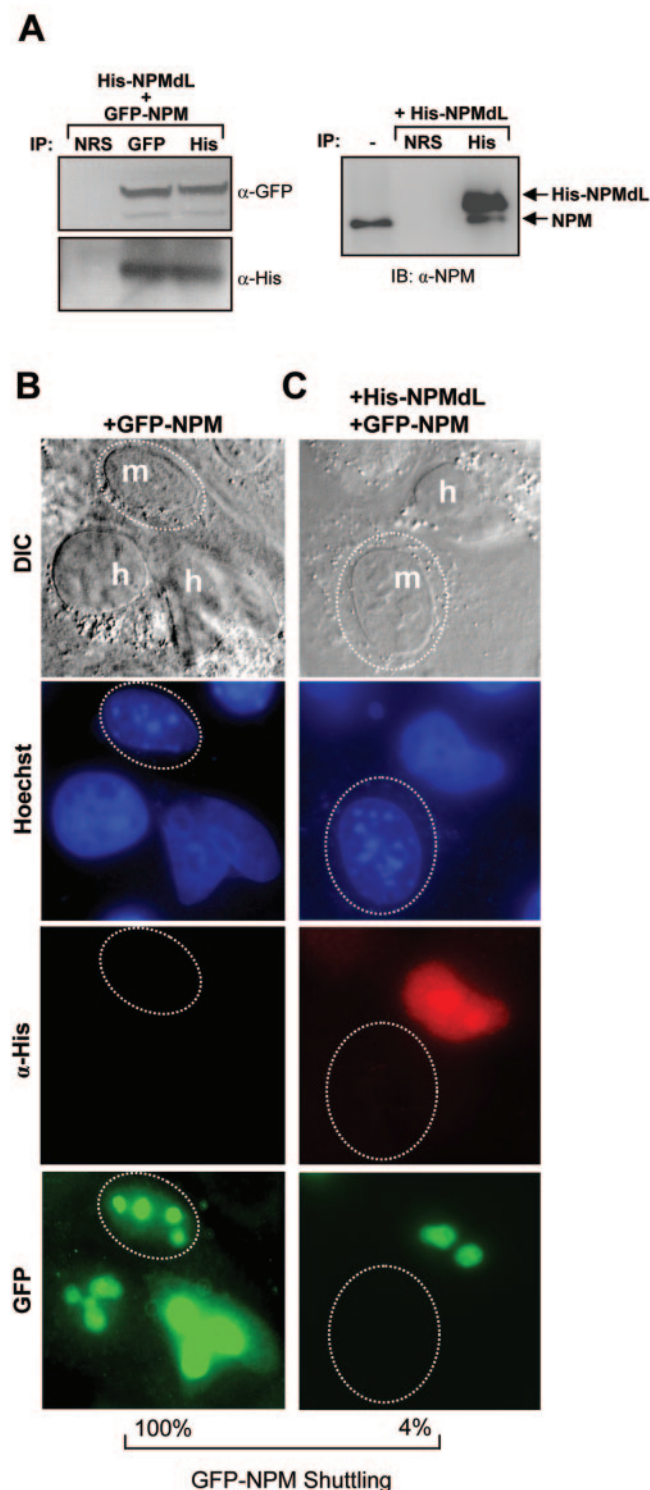


FIG. 3. NPM shuttling mutants act as dominant negative inhibitors of NPM nuclear export. (A, left) HeLa cells transfected with His-NPMdL and GFP-NPM were lysed, and the whole-cell lysate was subjected to immunoprecipitation (IP) with NRS or antibodies recognizing His and GFP epitopes. Precipitated protein complexes were separated by SDS-PAGE, and ectopic NPM proteins were visualized with antibodies against GFP and His epitopes. (A, right) HeLa cells transfected with His-NPMdL were lysed, and the whole-cell lysate was subjected to immunoprecipitation with NRS or antibodies recognizing His epitopes. Precipitated protein complexes were separated by SDS-

inhibition), indicating that these two leucine residues are critical for nuclear export of the NPM protein (Fig. 2C). Sequence alignment of numerous nucleophosmin homologues underscores the evolutionary importance of this amino-terminal export motif as it is nearly identical from zebra fish to humans (Fig. 2D).

**Heterogeneous complexes containing NPM NES mutants and wild-type NPM fail to shuttle.** Because NPM readily self-oligomerizes (32, 33, 34, 53), we considered the possibility that mutant NPM molecules could form hetero-oligomers with wild-type NPM proteins. To test this hypothesis, HeLa cells were transduced with His-tagged NPMdL expression vectors. Immunoprecipitation of His-NPMdL proteins revealed the coprecipitation of wild-type endogenous NPM proteins, demonstrating the formation of mutant–wild-type hetero-oligomers in cells (Fig. 3A, right). Additionally, cells transduced with His-NPMdL and GFP-NPM displayed formation of hetero-oligomers, as observed by coprecipitation of both proteins using antibodies directed at either epitope tag (His or GFP; Fig. 3A, left). Given our finding that mutant NPM forms oligomers with wild-type NPM, we next examined whether the NPM shuttling mutant NPMdL could also block wild-type NPM from shuttling. In the absence of the shuttling mutant, GFP-tagged NPM readily shuttled from human to mouse nucleoli (Fig. 3C). However, in the presence of His-tagged NPMdL, GFP-NPM was retained in human nuclei (Fig. 3D; 96% inhibition). Although we were unable to determine the exact stoichiometry between mutant proteins and wild-type proteins in the NPM oligomer, it is clear that overexpression of NPMdL severely impaired the shuttling activity of nearly all NPM oligomers.

**NPM associates with cytoplasmic and nuclear rpL5 ribosome complexes.** Previous studies have indicated that NPM might function as an integral component of ribosome maturation through its RNA binding activities (36). However, most hypotheses in this regard are largely based on the fact that NPM is nucleolar and, thus, most likely to be involved in the major process in the nucleolus: ribosome biogenesis. To formally test the nucleolar function of NPM, we examined the composition of *in vivo* NPM protein complexes in HeLa cell lysates. We generated a custom NPM polyclonal antibody affinity column and used a control nonimmune immunoglobulin column to preclear protein lysates. NPM complexes were eluted with increasing salt concentrations and visualized following SDS-PAGE and SYPRO-Ruby staining. As seen in Fig. 4A, we observed very little protein bound to our non-

PAGE, and ectopic mutant and endogenous wild-type NPM proteins were visualized with antibodies against NPM. Untransfected HeLa whole-cell lysate was loaded as a marker for endogenous NPM expression (lane 1). IB, immunoblot; α, anti; DIC, differential interference contrast. NIH 3T3 cells were seeded onto HeLa cells that had been transfected with GFP-NPM (B) alone or (C) in combination with His-NPMdL. Heterokaryon assays were performed, and His-NPMdL and GFP-NPM proteins were visualized with antibodies against His (red) or naturally emitting GFP spectra (green). DNA was stained with Hoechst. Mouse nuclei are demarcated with dotted circles. Human and mouse nuclei are labeled h and m, respectively. These data are representative of at least five independent heterokaryons formed for each transfection condition in three independent experiments. The percentages of GFP-NPM shuttling in heterokaryons are given.



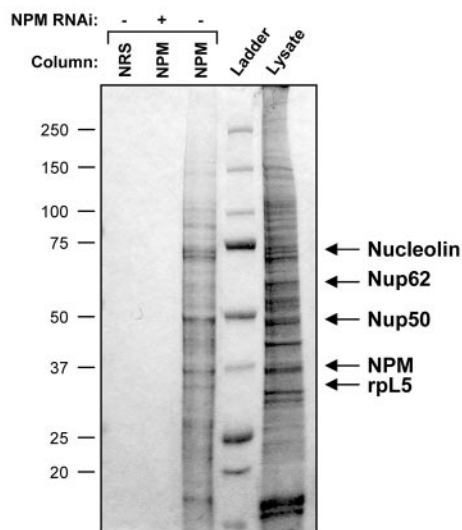
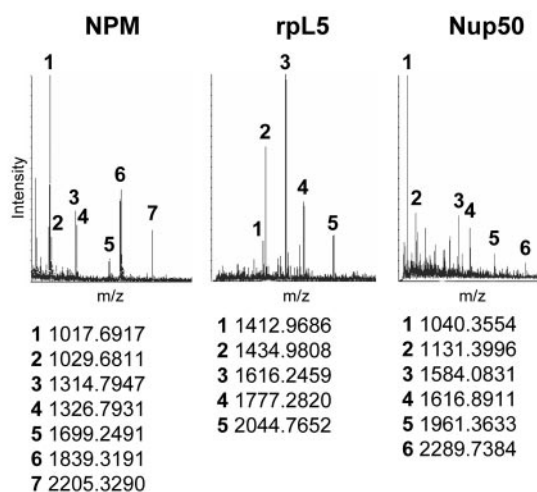
**A****B**

FIG. 4. Isolation of endogenous NPM protein complexes. (A) HeLa cell lysates (600  $\mu$ g) transduced with control (–) or NPM-directed RNA interference (RNAi) constructs (+) were injected onto either an NRS column or custom NPM polyclonal antibody affinity columns and eluted with an increasing NaCl gradient (0.1 to 1.0 M). Eluted proteins were separated by SDS-PAGE and visualized with Coomassie blue stain. Identified bands are labeled. (B) Representative MALDI-TOF spectra of labeled protein bands from panel A are shown with labeled matching peptide masses.

immune rabbit serum column (lane 1). However, some proteins (~18) were specifically eluted from the NPM antibody column (lane 3), including NPM and the previously known binding protein nucleolin. To determine whether the eluted proteins were in fact bound to the column through their interaction with NPM, we depleted NPM from HeLa cells using NPM-targeted RNA interference. Knockdown of NPM resulted in a loss of specific proteins bound and eluted from the

NPM antibody column, demonstrating that our identified NPM protein complex is specific for NPM (lane 2). Protein bands were excised and identified using MALDI-TOF and tandem mass spectrometry analyses. Among those proteins bound to NPM, a cluster of proteins associated with ribosome biogenesis, including rpL5 and nucleolin, as well as the nuclear pore complex proteins Nup50 and Nup62, were identified (Fig. 4A and B), with nucleolin (C23) being the only known NPM binding protein (26, 27). Western blot analysis of NPM protein complexes verified the presence of these proteins in salt-eluted fractions (data not shown).

Given the novelty and potentially significant ribosome biology of finding rpL5 in the NPM complex, we focused on verifying the NPM-rpL5 interaction. Purified recombinant NPM, NPMdL, and rpL5 proteins (Fig. 5A, left panels) mixed overnight were coprecipitated (Fig. 5A, middle panels), demonstrating that the NPM-rpL5 interaction is direct and independent of the NPM nuclear export signal. To show that the interaction of recombinant proteins was specific, NPM and rpL5 were mixed overnight with recombinant p27kip1 proteins (equally charged proteins not bound to the NPM antibody column). Precipitated proteins exhibited no complex formation between NPM and p27kip1 or rpL5 and p27kip1 (Fig. 5A, right panels). Both NPM and rpL5 readily interact with RNAs through conserved nucleic acid binding domains. To determine whether RNA binding is required for the NPM-rpL5 interaction, HeLa lysates were subjected to RNase A treatment prior to coprecipitation of NPM-rpL5 complexes. Even in the presence of RNase A, NPM and rpL5 visibly formed in vivo protein complexes (Fig. 5B) indistinguishable from those from untreated cells and consistent with our earlier finding that the interaction can be recapitulated with purified recombinant proteins (Fig. 5A). While NPM and rpL5 formed complexes in vivo, serial immunoprecipitation of NPM proteins from HeLa lysates showed that NPM and rpL5 are not exclusive partners. We failed to detect rpL5 in some NPM complexes (Fig. 5C, 3° and 4°), and we also noted that there was a significant amount of rpL5 free from NPM complexes in the remaining supernatant (Fig. 5C, Sup), indicating that both NPM and rpL5 can exist in complexes independent of one another.

Having identified a critical member of the 60S ribosomal subunit, namely, rpL5, in NPM complexes, we wanted to evaluate the colocalization of NPM with ribosomes in vivo. In order to follow the spatial control of NPM-rpL5 complexes in vivo, we utilized the UV absorbance of the ribosome. Ribosomal protein L5 is known to supply the maturing 60S ribosomal subunit with 5S rRNA prior to nucleolar/nuclear export of the 60S subunit (47), providing NPM an ideal time to form nucleolar complexes with rpL5. Cytoplasmic and nuclear extracts of HeLa cells were subjected to sucrose gradient centrifugation, and the gradients were fractionated with continuous UV monitoring. As shown in Fig. 6, NPM associates with the 40S, 60S, 80S, and polysome fractions in the cytoplasm while nuclear pools of NPM associate with the 40S/pre-60S and 60S fractions in the nucleus. Consistent with previous reports (30), we found rpL5 associated with the 60S, 80S, and polysome fractions in the cytoplasm and the 40S/pre-60S and 60S fractions in the nucleus (Fig. 6). These data demonstrate that NPM and rpL5 are localized with the maturing 60S ribosomal subunits in the nucleus and are maintained in the mature ribo-

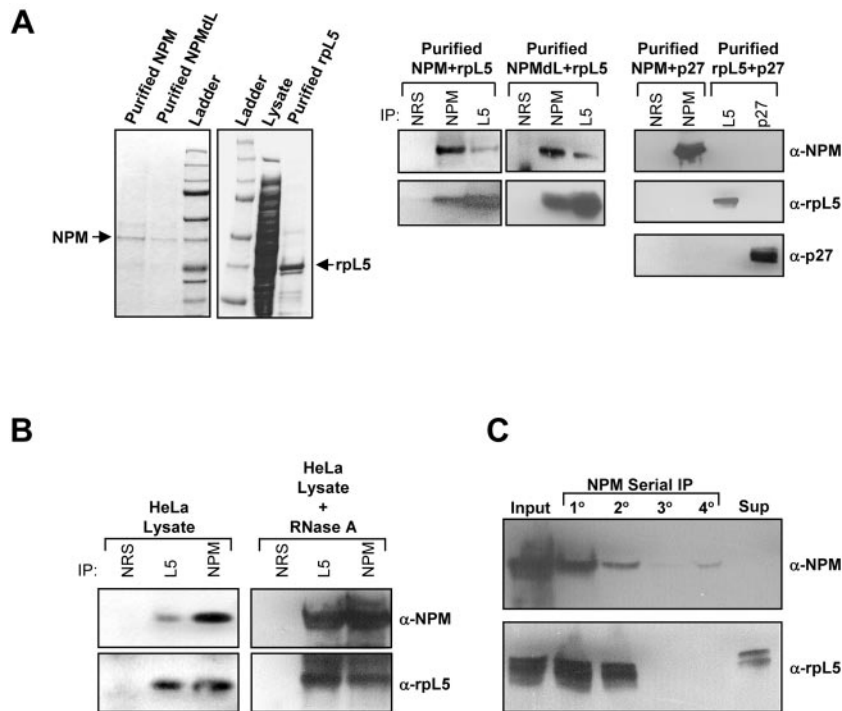


FIG. 5. NPM interacts directly with rpL5. (A, left) Recombinant NPM, NPMdL, and rpL5 were purified from bacterial lysates using Ni-nitrilotriacetic acid affinity chromatography. Purified proteins were separated by SDS-PAGE and detected with Coomassie blue stain. (A, middle) Purified NPM or NPMdL proteins were incubated overnight with rpL5 and immunoprecipitated (IP) with NRS or antibodies recognizing NPM or rpL5. Precipitated proteins were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with NPM and rpL5 antibodies. (A, right) Purified NPM or rpL5 proteins were incubated overnight with recombinant p27 and immunoprecipitated with NRS or antibodies recognizing NPM, rpL5, or p27. Precipitated proteins were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with NPM, rpL5, and p27 antibodies.  $\alpha$ , anti. (B) Proteins from HeLa cell lysates were immunoprecipitated with NRS, rpL5 antibody, or NPM antibodies. Precipitated proteins were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with NPM and rpL5 antibodies. Alternatively, HeLa lysates were pretreated for 1 h with RNase A prior to immunoprecipitation as described above. (C) HeLa lysates were subjected to serial immunoprecipitation with NPM antibodies (lanes 1<sup>o</sup> to 4<sup>o</sup>). Precipitated proteins and proteins in the final supernatant (unbound) were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with antibodies recognizing NPM and rpL5.

some once it reaches the cytosol. They also indicate that NPM also associates with the 40S subunit, which is devoid of rpL5 (Fig. 6) (30).

Transduction of HeLa cells with His-NPMdL resulted in a dramatic redistribution of rpL5 in cytosolic ribosomes; rpL5 was maintained in the 60S subunits but severely reduced in 80S ribosomes (Fig. 6, middle left panels). Given these findings, we cannot rule out the possibility that rpL5 proteins are still capable of some NPM-independent shuttling. However, it is more likely that rpL5 association with cytosolic ribosomes in the presence of NPMdL is a result of preexisting, stable cytosolic rpL5 complexes. This notion is further substantiated by treatment of HeLa cells with LMB. LMB treatment yielded results that were consistent with NPMdL overexpression (Fig. 6, lower panel). Both NPM and rpL5 proteins were found in the cytosol of LMB-treated cells (at reduced levels), even though the nuclear export of both proteins is LMB sensitive. This finding suggests that some preexisting cytosolic NPM and rpL5 ribosome complexes are fairly stable ( $\sim 24$  h) and that, if either protein utilizes CRM1-independent export, it is minimal.

**NPM is required for rpL5 nuclear export.** Having demonstrated a reduction of rpL5 associated with cytosolic ribosome subunits in the absence of NPM nuclear export signals, we next

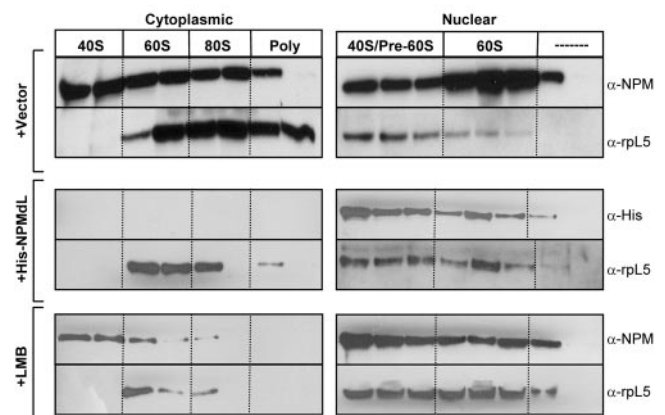


FIG. 6. NPM and rpL5 colocalize with nuclear and cytosolic ribosome subunits. HeLa cells transduced with vector (top panels) or His-NPMdL (middle panels) or treated with LMB (bottom panels) were divided into cytoplasmic and nuclear fractions and subjected to sucrose gradient centrifugation. Absorbance was monitored at 254 nm, and fractions containing 40S, 60S, 80S, and polysome units were collected. Proteins from each fraction were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with antibodies recognizing NPM, the His epitope, and rpL5.  $\alpha$ , anti.

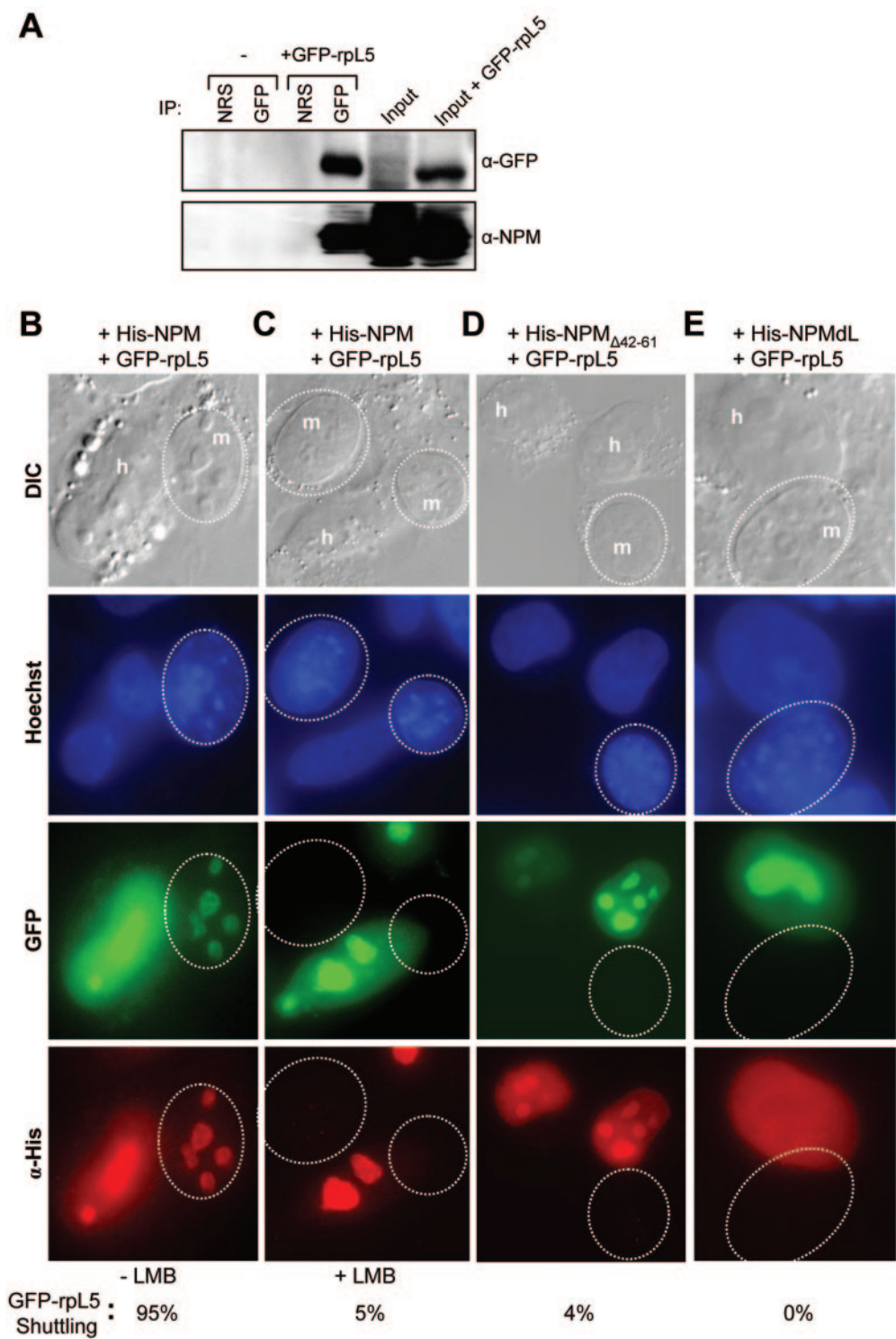


FIG. 7. NPM nuclear export signals are required for the efficient export of GFP-rpL5. (A) HeLa cells either untransfected or transfected with GFP-tagged L5 for 48 h were harvested and lysed. Proteins were immunoprecipitated (IP) with NRS or a rabbit GFP antibody. Precipitated proteins were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with GFP and NPM antibodies. α, anti; DIC, differential interference contrast. Loading inputs are indicated. (B to E) NIH 3T3 cells were seeded onto HeLa cells that had been transfected with GFP-rpL5 in combination with (B and C) His-NPM, (D) His-NPM<sub>Δ42-61</sub>, and (E) His-NPMdL. Additionally, HeLa cells in panel C were treated with LMB for 18 h prior to fusion. Heterokaryon assays were performed with NPM and GFP-rpL5 proteins being visualized with antibodies against His (red) and naturally emitting GFP spectra (green), respectively. DNA was stained with Hoechst. Mouse nuclei are demarcated with dotted circles. Human and mouse nuclei are labeled h and m, respectively. These data are representative of at least five independent heterokaryons formed in three independent experiments. The percentages of heterokaryons exhibiting GFP-rpL5 shuttling are given.



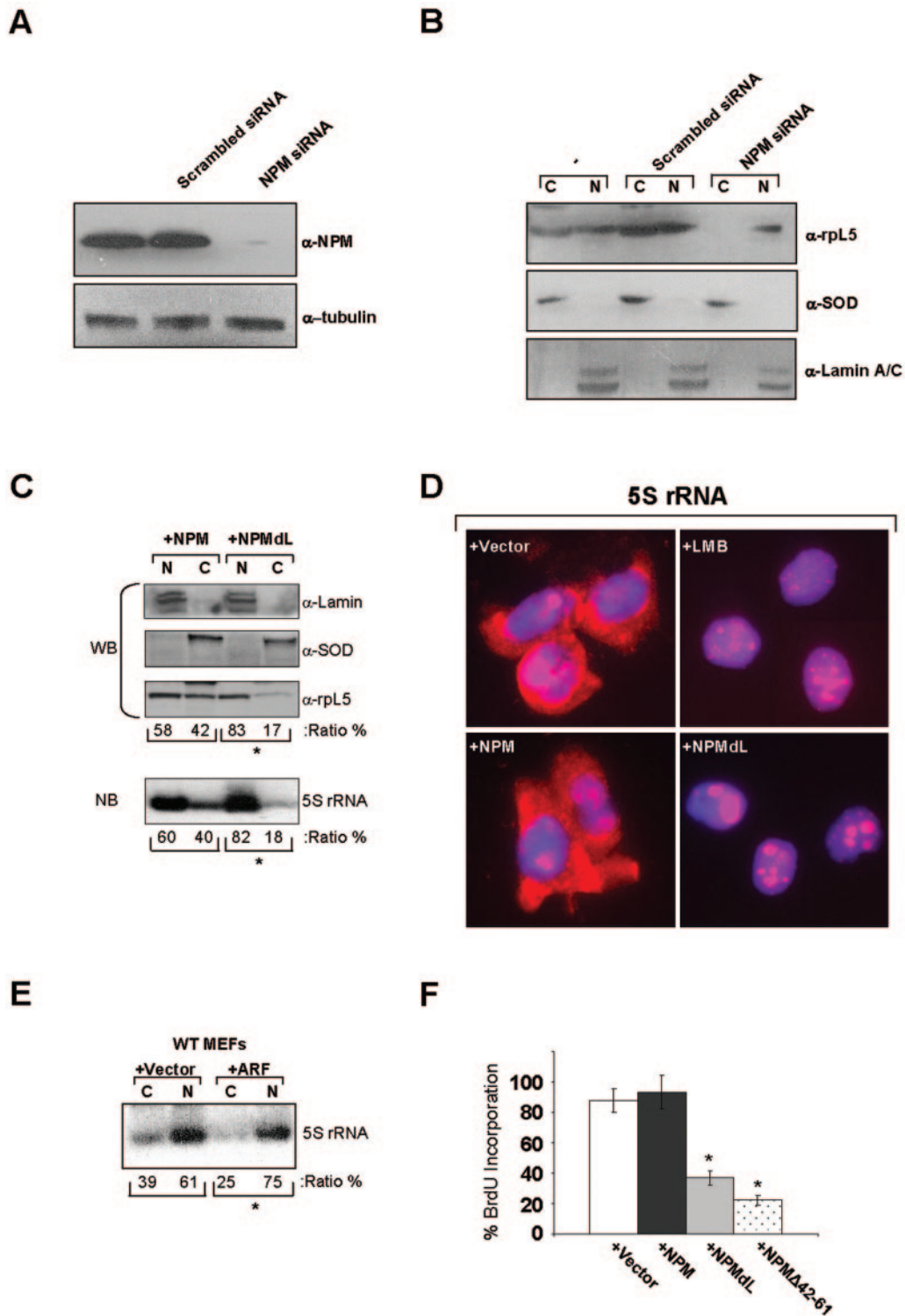


FIG. 8. NPM is essential for rpL5 nuclear export. (A) HeLa cells (–) or cells transduced with siRNAs encoding either scrambled control or NPM-specific sequences were harvested 72 h posttransduction for Western blot analysis. Proteins separated by SDS-PAGE were transferred to PVDF membranes and immunoblotted with antibodies recognizing NPM and  $\gamma$ -tubulin.  $\alpha$ , anti. (B) HeLa cells (–) or cells transduced with siRNAs encoding either scrambled control or NPM-specific sequences were harvested 72 h posttransduction for cellular fractionation. Proteins from nuclear (N) and cytosolic (C) fractions were analyzed by SDS-PAGE and immunoblotted with antibodies recognizing rpL5, SOD (cytoplasmic control), and lamin A/C (nuclear control). (C) HeLa cells were transfected with His-NPM or His-NPMdL, and 24 h later equal numbers of cells were subjected to fractionation into cytoplasmic (C) and nuclear (N) extracts. L5 protein was detected by Western blot analysis (top panels; WB). Lamin A/C and SOD are shown as nuclear and cytoplasmic fractionation controls, respectively (top panels; WB). 5S rRNA was detected by Northern blot analysis of total RNA extracted from the nuclear and cytosolic fractions (bottom panel; NB). The ratios of nuclear to cytoplasmic

examined the direct influence of NPM shuttling mutants on rpL5 nuclear export using a previously characterized GFP-tagged rpL5 protein (41). To confirm that GFP-rpL5 retained the NPM-binding properties of the endogenous rpL5 protein, we transiently overexpressed GFP-rpL5 in HeLa cells and performed Western blot analysis of GFP-immunoprecipitated complexes. As shown in Fig. 7A, precipitated GFP-rpL5 complexes contained endogenous NPM, confirming that the GFP moiety does not adversely affect the formation of NPM-rpL5 complexes *in vivo*. GFP-rpL5 and His-NPM readily migrated from human nucleoli to mouse nucleoli, as visualized in interspecies heterokaryons (Fig. 7B). However, in the presence of LMB, both GFP-rpL5 and His-NPM failed to shuttle (95% inhibition; Fig. 7C). Introduction of NPM shuttling mutant NPM $_{\Delta 42-61}$  or NPMdL inhibited GFP-rpL5 shuttling into mouse nucleoli, restricting its expression to human nuclei (Fig. 7D and E; 96% and 100% inhibition, respectively), establishing that NPM nuclear export is required for the export of rpL5. To more definitively show that NPM is required for rpL5 nuclear export, we knocked down NPM expression in HeLa cells (Fig. 8A). Cells lacking NPM protein expression failed to accumulate rpL5 in the cytoplasm, while cells transduced with scrambled small interfering RNA (siRNA) as a control exhibited an equal distribution of rpL5 between the nucleus and cytoplasm (Fig. 8B). These data underscore the necessity of NPM proteins for the efficient transport of rpL5 out of the nucleus and into the cytoplasm.

Ribosomal protein L5 is known to bind specifically to the mature 5S rRNA and aid in its nucleocytoplasmic transport (31, 37, 47). We hypothesized that NPM export, through its influence on rpL5, is the critical determinant for 5S rRNA nuclear export. To test this hypothesis, we performed Northern blot analysis of similar cellular fractions in the presence and absence of NPM shuttling. Indeed, in the presence of dominant negative NPM shuttling mutants, 5S rRNA failed to accumulate in the cytosol and instead was retained in the nucleus in a ratio similar to that for rpL5 (Fig. 8C). In addition to our fractionation studies, we performed RNA FISH to visualize the localization of steady-state levels of 5S rRNA. As shown in Fig. 8D, 5S rRNA was distributed throughout the nucleoli/nuclei and cytoplasm of HeLa cells transduced with empty vector as well as with wild-type NPM. Consistent with our fractionation data, inhibition of NPM and rpL5 nuclear export with LMB or NPMdL resulted in a severe attenuation of 5S rRNA export to the cytosol (Fig. 8D).

To further expand on this theme, we transduced wild-type MEFs with ARF, a known inhibitor of NPM nuclear export (7). Again, in the presence of the ARF tumor suppressor, 5S

rRNA failed to transit to the cytosol and instead was retained in the nucleoplasm (Fig. 8E). These data imply that ARF and NPM mutants defective in shuttling act similarly to prevent rpL5-5S rRNA nuclear export. To determine whether NPM shuttling mutants also prevent cell cycle progression, HeLa cells transduced with NPM expression constructs were labeled with 5-bromodeoxyuridine (BrdU) to measure active DNA synthesis. Similar to ARF's known cell cycle arrest properties (7), cells expressing NPMdL or NPM $_{\Delta 42-61}$  failed to enter S phase (Fig. 8F). Thus, NPM shuttling activity is not only required for the nuclear export of the rpL5-5S rRNA complex but also necessary for continued cell proliferation.

## DISCUSSION

The nucleolus, a highly specialized and structured organelle, has been described as the cell's control center for ribosomal synthesis, maturation, and assembly, with a host of proteins, RNAs, and other factors being implicated in these processes (8). Recently, numerous proteins (cdcl4, NPM, cyclin E, Mybbp1a, telomerase reverse transcriptase, and others) have been shown to continuously shuttle from the nucleolus to various subcellular compartments in a regulated manner, providing evidence that the nucleolus is a dynamic site of multiple cellular events (4, 7, 21, 22, 52).

One such protein, NPM/B23, has been linked to a variety of important cellular processes, both in and out of the nucleolus, including ribosome processing, molecular chaperoning, maintenance of genomic integrity, centrosome duplication, and transcriptional regulation (9, 10, 13, 16, 23, 35). Initially, NPM which was imported into the nucleolus from the cytoplasm was presumed to move about the various compartments of the nucleus (6), a feature shared by many critical cell cycle regulators. This shuttling of proteins between the nucleus and cytoplasm is now recognized as a key mechanism for ensuring proper cell cycle progression (39, 43). In previous reports, we and others identified NPM as a novel p53-independent target of the ARF tumor suppressor protein (5, 7, 19). We have since shown that, in response to hyperproliferative signals, nucleolar ARF directly binds NPM, effectively inhibiting NPM's nucleocytoplasmic shuttling. Here, we have further explored the mechanism and significance of NPM intracellular trafficking. First, we have described the CRM1-dependent nuclear export of NPM, identifying the two leucine residues (42 and 44) that are critical to this process. In addition, we have shown that alteration of the NPM NES resulted in the failure of wild-type NPM to be exported out of the nucleolus, providing evidence that these mutations function in a dominant-negative fashion,

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accumulation of rpL5 and 5S rRNA are given as percentages of totals (\*,  $P > 0.001$ ). (D) HeLa cells were transfected with vector, His-NPM, or His-NPMdL, and cells were plated on glass coverslips. Twenty-four hours later, cells were subjected to RNA FISH with a TRITC-labeled 5S rRNA probe. Nuclei were stained with DAPI. Untransfected HeLa cells were treated with LMB for 18 h prior to RNA FISH analysis. Results of 5S rRNA localization are each representative of three independent experiments. (E) Wild-type (WT) MEFs were infected with control retroviruses or those encoding ARF, and 48 h later equal numbers of cells were subjected to fractionation into cytoplasmic (C) and nuclear (N) extracts. 5S rRNA was detected by Northern blot analysis of total RNA extracted from the nuclear and cytosolic fractions (\*,  $P > 0.005$ ). (F) HeLa cells were transfected with vector, His-NPM, His-NPMdL, or His-NPM $_{\Delta 42-61}$  and plated on glass coverslips. Cells were incubated with BrdU 72 h posttransfection and fixed 20 h later. Fixed cells were stained with antibodies recognizing BrdU and His epitopes and visualized by immunofluorescence using fluorescein isothiocyanate- and TRITC-labeled secondary antibodies, respectively. Cells (100) were counted for each condition in triplicate. Standard deviations are reported as error bars (\*,  $P > 0.005$ ).

through the formation of NPM-NPMdL heteromultimers. Thus, NPMdL mimics the effects of ARF induction by directly impeding the nucleocytoplasmic shuttling of NPM through direct interaction, further demonstrating that NPM must exit the nucleolus/nucleus to maintain and promote cell growth.

We have previously proposed that targets of nucleolar sequestration might in fact "ride the ribosome" from the nucleolus to the cytoplasm to engage in growth-promoting events (45). In agreement with this hypothesis, our findings reveal a direct interaction between NPM and rpL5, providing the first physical link between NPM and ribosomal subunits. Much of the field's focus has been on the putative role of rpL5 in delivering 5S rRNA to the nucleolus, following the initial transcription of 5S rRNA by RNA polymerase III in the nucleoplasm (31, 37, 47). However, it is also possible that rpL5 is a critical player in the export of the large ribosomal subunit (60S), containing 5S rRNA, from the nucleolus/nucleus to the cytoplasm after its assembly. Clearly, the latter events would render themselves sensitive to NPM regulation, given that NPM provides the necessary export signals and chaperoning capabilities (via rpL5) required to transport components of the ribosome to the cytosol. Indeed, inhibition of NPM nuclear export via deletion or mutation of its NES prevented the trafficking of rpL5, an integral component of the 60S ribosomal subunit. Moreover, reduction of NPM expression through RNA interference completely abolished the cytosolic stores of rpL5, underscoring the absolute requirement for NPM in rpL5 nuclear export. Thus, our initial hypothesis of "riding the ribosome" should be revised to "taking the ribosome for a ride."

While many components of the ribosome, including rpL5, encode their own NESs, it is clear that a single NES forms a relatively weak interaction with CRM1 (25), suggesting a requirement for additional NESs in the efficient export of complexes. Consequently, proteins like NPM and NMD3 may have evolved to serve this purpose. Additionally, NPM and rpL5 were found, in reduced amounts, in cytosolic 40S and 60S complexes, respectively, after LMB treatment, implying either that these particular protein-ribosome complexes are fairly stable or that minor fractions of NPM and rpL5 utilize CRM1-independent modes of transport from the nucleus. Considering that the predominant function of rpL5 is to bind and mobilize 5S rRNA molecules, it was not surprising that 5S transport was also NPM sensitive, and thus NPM contributes to the efficient nuclear export of rpL5-5S rRNA complexes. However, NPM was present in 40S, 60S, 80S, and polysomes in the cytoplasm, implying that NPM, free (within the 40S subunit) or bound to rpL5, remains associated with the mature ribosome as it assembles and forms actively translating polysomes in the cytosol. Taken together, these findings open up the possibility that NPM might transmit additional cues (beyond nuclear export) to cytosolic ribosomes during translation, consistent with nucleolus's proposed role in dictating translation rates (28).

While it has been appreciated for several decades that changes in nucleolar structure are reliable markers of cellular transformation, experiments that provide a direct link between nucleolar dysfunction and tumorigenesis remain to be conducted. In fact, the nucleolus has largely been dismissed as a static organelle, having little to no impact on the overall well-being of the cell. However, this "nucleolar stigma" recently has been challenged with the discovery that tumor suppressors,

such as p53 and ARF, play a direct role in regulating nucleolar processes (5, 7, 42, 49). Interestingly, rpL5 is also a binding partner of Mdm2 and p53 (12, 17, 29), suggesting that rpL5 may provide an intriguing mechanistic link between ARF and ARF-binding partners. Clearly, through its interaction with NPM, ARF is capable of inhibiting nuclear export of rpL5-5S rRNA complexes. Inhibition of NPM-directed rpL5-5S nuclear export by ARF or NPM mutants defective in shuttling results in cell cycle arrest, demonstrating the importance of rpL5-5S export in maintaining cell proliferation. Moreover, NPM itself is a unique player in both the p53 and ARF responses (10, 11), providing us with a glimpse of how this network of protein interactions may inevitably become sensitive to oncogenic and tumor-suppressive signals in determining tumorigenic cell fates.

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## **Nucleophosmin directs ribosome nuclear export and cell growth**

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## Abstract

Nucleophosmin (NPM/B23) is an essential protein in mouse development and cell growth. NPM is a critical target of ARF tumor suppression while, in the absence of *ARF*, NPM is capable of transforming cells. Here, we present the first reported mechanism for NPM's regulation of cell growth; NPM directs the nuclear export of both 40S and 60S ribosomal subunits. Transduction of NPM shuttling-defective mutants or loss of *Npm*, inhibited nuclear export of both the 40S and 60S ribosomal subunits, reduced the available pool of cytoplasmic polysomes, and diminished overall protein synthesis without affecting ribosomal RNA processing. Modest increases in NPM expression amplified the export of newly synthesized ribosomal RNAs, resulting in increased rates of protein synthesis and indicating that NPM is rate-limiting in this pathway. These results support the idea that NPM-regulated ribosome export is a fundamental process in growth and cellular transformation.

## Introduction

The nucleolus is a dynamic subnuclear organelle that contains proteins not only involved in ribosome production but also oncogenesis and tumor suppression (Maggi and Weber 2005). As the site for ribosome biogenesis, a cell's nucleoli are centered around ribosomal DNA (rDNA) genes which are transcribed to produce ribosomal RNAs (rRNAs) (Warner 1990). A host of proteins are involved in the intricate processing and assembly steps required to produce the small, 40S, and large, 60S, ribosomal subunits (Warner 1990; Eichler and Craig 1994; Moss and Stefanovsky 2002). Once exported from the nucleus, the 40S and 60S subunits are combined in the cytoplasm to form functional 80S subunits (Johnson et al. 2002; Moss and Stefanovsky 2002). While the steps involved in the transcription and processing of rRNAs within the nucleolus are beginning to be understood, little is known about how ribosomal subunits are exported to the cytosol in mammalian cells. Studies from *Saccharomyces cerevisiae* and *Xenopus laevis* oocytes have shown that the process is dependent on the CRM1-RanGTP export receptor pathway (Johnson et al. 2002). Additionally, NMD3, a highly conserved adapter protein, assists in the nuclear export of the 60S subunit in yeast (Ho and Johnson 1999). However, experiments in mammalian cells demonstrated that nuclear export of rRNAs was not completely dependent on NMD3, with some rRNAs not affected at all (Trotta et al. 2003). Thus other nucleolar proteins must be involved in the nuclear export of ribosomes.

The difficulty in assessing nucleolar protein function stems from early assumptions that all nucleolar proteins must be involved, in some way, with rDNA transcription or rRNA processing by virtue of their unique subnuclear localization. However, a more contemporary view of the nucleolus as a dynamic nuclear organelle capable of regulating numerous cellular processes, apart from rDNA transcription and rRNA processing, has led to a re-evaluation of

nucleolar protein function(s). Nucleophosmin (NPM) is an abundant nucleolar phospho-protein that is highly expressed in proliferating cells (Feuerstein et al. 1988). Our lab and others have shown that NPM is a target of the nucleolar tumor suppressor, p19ARF (Itahana et al. 2003; Bertwistle et al. 2004; Brady et al. 2004). We have shown that NPM can shuttle from the nucleolus/nucleus to the cytoplasm, but upregulation of ARF can block its nucleolar/cytoplasmic trafficking (Brady et al. 2004). NPM shuttling requires a conserved CRM1 binding site and mutation of two critical leucine residues blocks NPM's ability to leave the nucleus (Yu et al. 2006). Additionally, NPM is an essential nucleolar protein with acute loss of expression resulting in severe attenuation of cellular proliferation and increased apoptosis (Itahana et al. 2003; Bertwistle et al. 2004; Brady et al. 2004). In agreement with these *in vitro* findings, two recent reports have shown that *Npm* is required for embryonic development and that fibroblasts derived from *Npm*-deficient embryos have a reduced capacity to proliferate (Colombo et al. 2005; Grisendi et al. 2005). Given NPM's reported involvement in numerous cellular processes as diverse as ribosome biogenesis (Okuwaki et al. 2002), centrosome duplication (Okuda et al. 2000), chaperoning (Okuwaki et al. 2001), and transcriptional control (Colombo et al. 2002), the underlying mechanism behind NPM's requirement for cellular proliferation has remained elusive.

In an effort to uncover this mechanism, we observed a consistent increase in cell size and proliferative capacity of fibroblasts overexpressing NPM that was dependent on the nuclear export activities of NPM. Furthermore, NPM trafficking led to amplified protein synthesis rates, underscoring the ability of NPM to drive growth and proliferation. Here, we describe the mechanism behind this observation, revealing a prominent role for NPM in driving ribosome nuclear export and thereby controlling cell growth.

## Results

### NPM is a Potent Oncogene in the Absence of ARF

NPM is induced upon mitogenic stimulation and is a direct transcriptional target of the *Myc* oncogene, providing the first evidence that NPM might be involved in cell cycle progression (Guo et al. 2000; Zeller et al. 2001; Menssen and Hermeking 2002; Watson et al. 2002). In order to further assess the role of NPM in promoting cell proliferation and transformation, we examined the effects of NPM overexpression on cell growth, S phase entry, and cellular transformation in immortalized *ARF*<sup>-/-</sup> mouse embryonic fibroblasts (MEFs) (Kamijo et al. 1997). As shown in Figure 1A, NPM overexpression induced a significant increase in cell size as measured by flow cytometry forward and side scatter, to a slightly greater extent than oncogenic Ras<sup>V12</sup>. *Arf*<sup>-/-</sup> MEFs transduced with either vector control or wild-type NPM readily enter S phase, as evidenced by incorporation of BrdU into replicating DNA (Figure 1B). In contrast, an NPM shuttling mutant, NPMdL (Yu et al. 2006), significantly attenuated S phase entry (Figure 1B), implying that NPM nuclear export is required for S phase entry. We next assessed the ability of NPM to transform immortalized *Arf*<sup>-/-</sup> MEFs in colony formation assays. Overexpression of NPM resulted in increased colony size in soft agar over control (Figure 1D, upper panels) although not as large as Ras<sup>V12</sup> colonies, as well as an increase in foci number (lower panels). Again, expression of the NPM shuttling mutant blocked colony growth in soft agar as well as foci formation, supporting the role of NPM nuclear export in these processes.

In order to further assess the role of NPM in promoting cell proliferation and transformation, we analyzed 60 carcinoma samples from breast, prostate and colon cancers. Approximately 10-18 % of the actively proliferating tumor samples (Ki-67 positive) stained negative for NPM (Figure 1B, left panels), indicating that not all highly proliferative tumor cells

engage in NPM overexpression as a readout of active cell cycle progression. The remainder of the samples stained positive for NPM (82-90%) with nearly 50% of the NPM-positive tumor samples, irregardless of tumor type, exhibiting particularly strong nuclear/nucleolar NPM staining (Figure 1D, right panels).

*Arf*<sup>-/-</sup> MEFs, although immortal, remain diploid (Kamijo et al. 1997) and retain normal centrosome numbers throughout cellular passaging *in vitro* (see below). Given previous findings that NPM plays a key role in centrosome duplication, we next sought to determine whether introduction of exogenous NPM altered the ploidy of *Arf*<sup>-/-</sup> cells through centrosome amplification. Additionally, Grisendi and colleagues recently showed that *Npm* loss results in multiple centrosome formation accompanied by genome instability, suggesting that NPM is a key regulator in maintaining proper centrosome duplication (Grisendi et al. 2005). As shown in Figure 1E, NPM overexpression did not alter centrosome number compared to control cells nor did it alter the overall chromosome number in *Arf*<sup>-/-</sup> MEFs (Figure 1F). Taken together these data demonstrate that the transforming properties of NPM are not coupled to its ability to regulate or dysregulate centrosome numbers and DNA ploidy.

### **Endogenous NPM Protein Complexes Contain Ribosomal Proteins**

In an effort to conduct an unbiased assessment of NPM function in promoting growth and proliferation, we examined the composition of *in vivo* NPM protein complexes isolated from HeLa cells. Gel filtration and anion exchange chromatography of HeLa cell lysates revealed numerous distinct endogenous NPM complexes (Figure 3A and data not shown). Using native gel analysis of the sizing column fractions, we were able to determine that oligomeric NPM was present in protein complexes greater than 440 kDa (Figure 3A and data not shown). In an

attempt to identify the protein components of the NPM complex, we generated an NPM polyclonal antibody affinity column. Pooled NPM complexes eluted from the sizing column were cleared over an IgG column prior to passage over the NPM affinity column and eluted with increasing salt concentrations. Further separation of NPM protein complexes by two-dimensional electrophoresis aided in the positive identification of various NPM-associated proteins via mass spectrometry analysis (Figure 3B). Among those proteins bound to NPM, a cluster of proteins associated with ribosome biogenesis, as well as the nuclear pore complex proteins, Nup50 and Nup62, were identified (Figure 3B and Table 1). A complete list of NPM complex proteins is shown in Table 1. Notably, rpL5, nucleolin and Brca1 proteins identified from our isolated NPM complexes have previously been shown to interact with NPM, demonstrating the effectiveness of our approach to identifying NPM components (Liu and Yung 1999; Sato et al. 2004; Yu et al. 2006). Of the fifteen known proteins identified in our NPM complex, nine constitute integral components of the 40S and 60S ribosomal subunits. Additionally, six of the remaining nine NPM binding proteins are involved in translational regulation. Although we could not determine the exact stoichiometry of these complexes to one another, it was apparent that NPM protein complexes involved in some facet of ribosome biogenesis predominated.

### **NPM Nuclear Export is Required for Protein Synthesis**

Testing the hypothesis that NPM-ribosomal protein complexes play a role in promoting cell growth, we next investigated the potential function of NPM in protein synthesis. HeLa cells transduced with empty vector, wild-type NPM or NPMdL, were labeled with <sup>35</sup>S-Methionine for the indicated times and total trichloroacetic acid-precipitable proteins were counted for label

incorporation. Addition of the shuttling defective mutant, NPMdL, significantly attenuated the rate of total protein synthesis. Interestingly, slight overexpression of wild-type NPM (Figure 4A inset) resulted in a significant increase in the rate of protein synthesis. This was not a result of simple acceleration of cell cycle progression as HeLa cells expressing exogenous NPM proliferated at a rate similar to control cells (data not shown).

We next examined NPM's ability to influence cytosolic ribosome profiles. Cytosolic ribosomes were isolated via sucrose gradient centrifugation from equal numbers of HeLa cells transduced with empty vector, wild-type NPM or NPMdL. Continuous evaluation of the gradient by ultraviolet absorbance for the presence of rRNAs indicated that in the absence of NPM shuttling (NPMdL), formation of cytosolic 40S and 60S ribosome subunits was dramatically impaired (Figure 4B, green line) while mild overexpression of NPM (Figure 4B, inset) resulted in enhanced formation of cytosolic 40S and 60S subunits (Figure 4B, blue line). Notably, NPM overexpression amplified (~30%) the amount of cytosolic ribosomes engaged in active mRNA translation (Figure 4B, blue line) in a mechanism that was dependent on the nuclear export activities of NPM molecules (Figure 4B, green line).

### **NPM is the Rate-limiting Factor for rRNA Nuclear Export and Cytosolic Ribosome Accumulation**

We have presented data here that NPM nuclear export is required for the enhanced accumulation of cytosolic ribosomes and polysomes (Figure 4). To understand the mechanism behind this observed increase in cytosolic ribosomes, we examined the effects of NPM on the nuclear export of newly synthesized rRNAs. HeLa cells transduced with control vector, wild-type NPM, or the shuttling mutant, NPMdL, were pulsed with [methyl-<sup>3</sup>H] methionine to label

newly synthesized and processed rRNAs (Sugimoto et al. 2003). Importantly, ectopic expression of wild-type NPM or NPMdL shuttling mutants had no effect on HeLa cell proliferation at the 24h time point used for these experiments (data not shown), allowing us to separate cell cycle progression from ribosomal biogenesis.

Consistent with our earlier findings for protein synthesis and ribosome profiles, NPMdL significantly attenuated (>12-fold) the cytoplasmic accumulation of all rRNAs to nearly undetectable levels (Figure 5A). In contrast, cells overexpressing wild-type NPM exhibited a significant increase (>7-fold) in the cytoplasmic accumulation of rRNAs (Figure 5A). We observed similar effects of NPM overexpression in *Arf*<sup>-/-</sup> MEFs (Figure 5B), indicating that these effects of NPM overexpression are not cell type specific and corroborate our initial growth and transformation findings in *Arf*<sup>-/-</sup> cells. Importantly, overexpression of NPM did not alter rRNA processing of newly transcribed 47S rRNA into 28S, 18S, and 5.8S rRNA species (Figure 5C), implying that increased accumulation of rRNAs in the cytosol was not simply due to enhanced rRNA processing in the nucleolus.

To determine the distribution of newly exported rRNAs into cytosolic ribosomes and polysomes, we pulse-labeled cells with [methyl-<sup>3</sup>H] methionine and measured the amount of incorporated label in each sucrose gradient fraction containing 40S, 60S, 80S and polysomes. While NPM overexpression appeared to affect the steady-state levels of polysome production (~30%, Figure 4B), transduction of cells with wild-type NPM greatly increased (~3-fold) the amounts of newly exported cytosolic ribosomal subunits, including the 40S, 60S, 80S and polysomes available for protein synthesis (Figure 5D), underscoring the ability of NPM to not only enhance the export of ribosomal subunits, but also to provide more ribosomes for polysome-directed protein synthesis.



### **Loss of NPM Blocks rRNA Export *in vitro* and *in vivo***

Inhibition of NPM shuttling activity using the dominant negative mutant, NPMdL, indicates that NPM nuclear export is required for rRNA export to the cytoplasm. However, we cannot rule out the possibility that the NPMdL mutant prevents ribosome export in an indirect and passive manner. To confirm that NPM is required for the nuclear export of rRNAs, we examined the effects of loss of NPM on export of newly synthesized rRNAs. HeLa cells transduced with scrambled control or NPM-specific short interfering RNAs (siRNAs) (Yu et al. 2006) were pulsed with [methyl-<sup>3</sup>H] methionine to label newly synthesized and processed rRNAs. Specific knockdown of endogenous NPM was confirmed (Figure 6A, bottom panel). Dramatic reduction of NPM protein expression significantly attenuated the cytoplasmic accumulation of all rRNAs to nearly undetectable levels (Figure 6A, top panel). In addition, transduction of HeLa cells with NPM siRNAs significantly decreased the steady state levels of all cytosolic ribosomal subunits (40S, 60S, 80S and polysomes) compared to scramble control (Figure 6B), providing strong evidence that NPM is an essential component in the nuclear export of ribosomal subunits and maintenance of sufficient pools of cytosolic polysomes. Similarly, MEFs carrying hypomorphic NPM alleles (*Npm*<sup>hy/hy</sup>), which express nearly undetectable levels of NPM ((Grisendi et al. 2005) and Figure 6C, bottom panel), show a striking decrease in nuclear export of 28S and 18S rRNA compared to littermate matched wild-type MEFs (Figure 6C).

Embryos nullizygous for *Npm* fail to develop properly and die *in utero* between days 11.5 and 16.5 (Grisendi et al. 2005). To determine whether a defect in rRNA nuclear export might be responsible for the growth retardation observed in these *Npm*-null embryos, we examined *in vivo* 5S rRNA export. Utilizing a biotinylated 5S rRNA probe, we performed RNA *in situ*

hybridization on day E10.5 wild-type and *Npm*<sup>-/-</sup> embryos. Littermate matched wild-type embryos readily exported 5S rRNA (purple stain) out of the nucleus (pink stain) and into the cytoplasm in numerous embryonic cell compartments (Figure 7A-D). In contrast *Npm*<sup>-/-</sup> embryos did not export 5S rRNA in any of the embryonic cells as evidenced by the accumulation of dark nuclear 5S RNA stain (purple) and lack of cytoplasmic stain (Figure 7E-H). Taken together these data indicate that NPM is absolutely required for export of rRNAs *in vivo*.

## Discussion

The nucleolus is a dynamic subnuclear organelle tasked with producing the core of the protein translation machinery of a cell: the ribosome (Warner 1990; Moss and Stefanovsky 2002). Historic models of nucleolar function have depicted ribosome biogenesis as a passive, reactionary process in the cell where growth signals are relayed to the nucleolus to increase ribosomal RNA (rRNA) synthesis, resulting in higher rates of ribosome production to meet the demands of increased proliferation rates. However, a more current view of the nucleolus as a sensor of both stress and growth has emerged. In challenge of this “static ribosome factory” model, numerous proteins originally thought to solely reside in the nucleolus have been recently shown to continuously shuttle from the nucleolus to various subcellular compartments in a regulated manner, providing evidence that the nucleolus is a dynamic site of numerous cellular events (Juan and Cordon-Cardo 2001; Keough et al. 2003; Azzam et al. 2004; Brady et al. 2004; Yu et al. 2006). One of these nucleolar proteins, NPM, has been suggested to be involved in a variety of important cellular processes in and out of the nucleolus including ribosome processing, molecular chaperoning, centrosome duplication, and transcriptional regulation (Feuerstein et al. 1988; Chan et al. 1989; Kondo et al. 1997; Okuda et al. 2000; Okuwaki et al. 2001; Okuwaki et al. 2002). Our current study offers data which integrates many of these seemingly disconnected functions of NPM.

One of the conundrums surrounding NPM is its apparent ability to perform functions that are in stark contrast to one another. Specifically, NPM has been reported to interact with all three components of the ARF-p53-Mdm2 pathway to both activate and suppress this cascade (Szebeni and Olson 1999; Colombo et al. 2002; Itahana et al. 2003; Bertwistle et al. 2004; Brady et al. 2004; Kuo et al. 2004; Kurki et al. 2004; Li et al. 2004). However, the overarching theme

of this pathway is the regulation of growth and proliferation. In this regards, we have shown that NPM can transform immortalized *Arf*<sup>-/-</sup> MEFs through increased protein synthesis rates, indicating, at the very least, that NPM is a growth promoter. Taken as single observations, numerous conflicting views of NPM function can be readily made. However, when placed together with our current findings, a more unified outlook on NPM function emerges as we will discuss below.

The mechanism of NPM's growth promoting properties is thought to be mandated through alterations in rRNA processing (Savkur and Olson 1998). We have clearly shown that NPM does not alter processing. In fact, we have presented evidence that NPM serves as a nuclear export chaperone for the ribosome, drawing similarities to earlier studies that showed an *in vitro* propensity for NPM to serve as a protein chaperone (Szebeni and Olson 1999; Okuwaki et al. 2001). Even modest increases in NPM protein expression amplified ribosome export resulting in increased rates of protein synthesis and ultimately cell growth, demonstrating that NPM is rate-limiting in its capacity to shuttle ribosomes to the cytosol. Under this model, NPM would serve as an adaptor, analogous to NMD3, bridging the ribosome with CRM1 prior to export through the nuclear pore (Trotta et al. 2003). In fact, we were able to co-purify nuclear pore proteins Nup50 and Nup62 with NPM demonstrating that these export complexes exist *in vivo*.

The similarities between NPM and NMD3 are intriguing, but there are enough measurable differences to suggest that they do not perform identical functions and rather most likely, complement one another in their ability to act as nuclear export chaperones. Unlike NMD3, we found NPM in complexes with ribosomal proteins from both the small and large ribosome subunits. Additionally, mutation of the NMD3 NES results in only an attenuation of

the 28S rRNA (Trotta et al. 2003), whereas NPMdL readily inhibits the nuclear export of all newly synthesized 28S, 18S and 5S rRNAs, suggesting that NPM may reside upstream of NMD3 in regulating the nuclear export of all ribosomal subunits. Moreover, overexpression of NPM was capable of dramatically increasing ribosome nuclear export, a finding that has not been produced for NMD3. The dramatic sequestration of the entire ribosome in the nucleus by mutant NPM molecules is underscored further by the rapid depletion of newly assembled polysomes in cells unable to shuttle NPM protein complexes. Thus, we propose that NPM serves as a “growth thermostat” in the nucleolus, ready to respond to changes in the cell that might require more or less ribosome output for protein synthesis. Loss of *Npm* confirms this hypothesis, with *Npm*<sup>hy/hy</sup> and *Npm*<sup>-/-</sup> cells displaying severe attenuation of all rRNA export.

Given NPM's role in the nucleolar growth promoting pathway through regulating ribosome export, it is not surprising that several groups including our own have shown NPM to be a p53-independent target of the nucleolar tumor suppressor ARF (Itahana et al. 2003; Bertwistle et al. 2004; Brady et al. 2004). The fact that NPM promotes ribosome export in *Arf*-null cells leading to increased protein synthesis and cellular transformation, suggests that ARF itself is not the critical target of NPM as has been recently postulated (Korgaonkar et al. 2005). Instead, in order to maintain proper nucleolar function, ARF must be sensitive to abrupt changes in ribosome output, constantly monitoring the rate of ribosome synthesis and export by NPM. We tend to think of ARF as a sensor of hyperproliferative signals, such as those emanating from Myc and Ras (Palmero et al. 1998; Zindy et al. 1998; Groth et al. 2000), but both of these oncoproteins are quite capable of eliciting hyper-growth signals as well. Indeed, recent evidence has demonstrated that oncogenic myc translocates to the nucleolus in order to greatly increase ribosomal DNA synthesis (Arabi et al. 2005; Grandori et al. 2005), and under some settings,

ARF can act to antagonize nucleolar myc oncoproteins (Qi et al. 2004; Gregory et al. 2005). In order to maintain nucleolar integrity in the face of myc-induced increases in ribosomal RNAs, the cell would also require NPM to enhance the rate of ribosome nuclear export. In support of this notion, *Npm* is a direct transcriptional target of myc (Guo et al. 2000; Zeller et al. 2001; Menssen and Hermeking 2002; Watson et al. 2002). Accordingly, nucleolar ARF has evolved to monitor this process, and as such, is also a target of myc induction (Zindy et al. 1998). In human cancers, it then becomes advantageous to disrupt the normal regulation of this process. Under normal conditions, NPM is devoted to ribosome nuclear export and protein synthesis is maintained at a constant rate. We would predict that this nucleolar homeostasis is interrupted by two different signals. First, stress signals that alter nucleolar structures (Rubbi and Milner 2003) and de-localize NPM to the nucleoplasm where it binds to p53 or Mdm2 (Colombo et al. 2002; Kurki et al. 2004) would serve to not only activate the p53 pathway but also to divert NPM from its normal role in rRNA export. Dramatic decreases in protein synthesis rates are hallmarks of cellular stress outcomes (Pyronnet and Sonenberg 2001). Second, oncogenic growth signals that up-regulate NPM protein expression would also trigger the ARF checkpoint with accumulated nucleolar ARF proteins preventing the nuclear export of NPM-ribosome complexes. Thus, in the absence of an intact ARF checkpoint, NPM proteins are capable of amplifying the rate of rRNA export, resulting in dramatic unopposed increases in protein translation and eventual cell transformation recorded in this report.

## Materials and methods

### Cell Culture, Virus Production, and Nucleofection

HeLa cells and *Arf*<sup>-/-</sup> MEFs were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 2 mM glutamine, 0.1 mM non-essential amino acids, and 100 U penicillin and streptomycin. *Npm*<sup>+/+</sup> and *Npm*<sup>hy/hy</sup> litter matched MEFs were maintained in identical media with gentimycin. Virus production and infection of MEFs were carried out using retroviral helper and vector plasmids provided by Charles Sawyers (UCLA). HeLa cells (2x10<sup>6</sup>) were nucleofected with 2 µg of the indicated plasmids according to manufacturer's instructions (Amaxa Inc.).

### Plasmid Constructs

Retroviral vectors encoding full-length His-tagged murine NPM, NPMdL, and H-Ras<sup>V12</sup> are described elsewhere (Groth et al. 2000; Brady et al. 2004; Yu et al. 2006). His-epitope tagged NPM and His-NPMdL in pcDNA3.1 expression vectors have been previously described (Yu et al. 2006). Short-interfering RNAs against NPM and a scrambled control siRNA have been previously described (Yu et al. 2006).

### FACS Analysis

*Arf*<sup>-/-</sup> MEFs infected with control, His-NPM, His-NPMdL, or Ras<sup>V12</sup> retroviruses were harvested 72 h post-infection. Cells were fixed in 95% ethanol, washed and resuspended in 1X PBS/1% FBS, and filtered prior to cell size analysis using a FACSCalibur flow cytometer (Becton Dickinson).

**Western Blot Analysis**

Cells were lysed by sonication in EBC buffer (25 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40) at the indicated times. NPM, Lamin A/C, SOD, and His-tagged proteins were visualized by direct immunoblotting with NPM (Zymed), Lamin A/C (Santa Cruz), SOD (Santa Cruz), and His (Santa Cruz) antibodies, respectively. As a loading control, blots were probed with  $\gamma$ -tubulin antibody (Santa Cruz) as indicated.

**Foci formation assay**

*Arf*<sup>-/-</sup> MEFS were infected with control, His-NPM, His-NPMdL, or Ras<sup>V12</sup> retroviruses and seeded ( $2 \times 10^3$ ) onto 100 mm dishes. Cells were grown for 14 days in complete medium, fixed in 100% MeOH and stained for 30 min with 50% Giemsa (vol:vol in H<sub>2</sub>O).

**Soft Agar Colony formation assay**

*Arf*<sup>-/-</sup> MEFS were infected with control, His-NPM, His-NPMdL, or Ras<sup>V12</sup> retroviruses and seeded ( $1 \times 10^3$ ) in quadruplicate wells of a 24 well plate. Colonies were allowed to grow for 14 days in complete media supplemented with FBS and Noble Agar.

**Common Cancer Tissue Array**

The TARP4 tissue array was purchased from NCI Tissue Array Research Project. The tissues used to construct arrays were obtained from the Cooperative Human Tissue Network (CHTN). Tissue array slides were mounted on siliconized glass slides and purchased ready for use in immunohistochemistry.



## **Immunohistochemistry**

Immunohistochemistry was performed using the SABC method on a BioGenex i6000<sup>TM</sup> automated staining system (BioGenex, San Ramon, CA). Briefly, de-paraffinized tissue sections were first treated with 3% H<sub>2</sub>O<sub>2</sub> for 30 min to inhibit endogenous peroxidase followed by antigen retrieval by microwave heating in citra plus solution (BioGenex) for 15 min. After subjecting to avidin block, biotin block and power block for 15 min, respectively, the sections were incubated with mouse anti-NPM (Zymed) primary antibodies as described above for 1 h at room temperature with an antibody dilution of 1:50. After further incubation with biotinylated multi-link antibody for 45 min and peroxidase-labeled strepavidin for 30 min, the staining was developed by reaction with 3,3'-diaminobenzidine tetrahydrochloride substrate-chromogen solution for 3 min followed by counterstaining with hematoxylin for 10 sec.

## **Semi-quantitative assessment of NPM expression in human tumors**

NPM signal was localized to the nucleolus and nucleus. The final staining scores were according to the extent of positive cells. 0-5% was scored as negative (-), 6-25% (+), 26-50% (++), 51-75% (+++). Cells exhibiting NPM staining (-) or (+++) are shown in the representative figure panel.

## **Fluid Phase Liquid Chromatography**

HeLa cells were lysed in Tween-20 lysis buffer (10 mM Tris-HCl, pH 7.4; 150 mM NaCl; 0.1% Tween-20; 1  $\mu$ M NaVO<sub>4</sub>; 10  $\mu$ M NaF; 1 mM PMSF; 1  $\mu$ g/ml Aprotinin) by sonication. Lysates (600  $\mu$ g) were injected on to a HiPrep 16/60 Sephacryl S-300 Column (Amersham). Proteins were eluted with 150 mM NaCl, 50 mM NaH<sub>2</sub>PO<sub>4</sub> pH 7.2 using BioLogic fluid phase liquid chromatography (FPLC) and HR software (Bio-Rad). Fractions were precipitated with

trichloroacetic acid (TCA), re-suspended in 1 M Tris-HCl (pH 7.4), separated by SDS-PAGE, transferred to PVDF membranes and immunoblotted with antibodies recognizing NPM (Zymed). For affinity chromatography, a rabbit polyclonal antibody recognizing the N-terminus of NPM (Sigma) was bound to NHS-activated Sepharose (Amersham). HeLa cells were lysed in 20 mM Tris pH 7.4, 0.1% Tween-20 and sonicated. Lysates (600 µg) were injected first over the sizing column and subsequent NPM pools were loaded onto the NPM affinity column, washed with 20 mM Tris and eluted with an increasing NaCl gradient (0.1-1M). Collected fractions were pooled, precipitated, and subjected to proteomic analysis as described below.

### **Proteomic Analysis**

Proteins from FPLC fractions were precipitated with TCA and resuspended in re-hydration buffer (BioRad). 500 µg of proteins and 12.5 µL of 200 mM tributylphosphine (BioRad) were mixed and loaded by passive in-gel re-hydration. IPG strips (BioRad, pH 3-10) were focused according to standard protocols. Separated proteins were stained with SYPRO-Ruby (BioRad). Tryptic peptides were calibrated with Sequazyme peptide mass standards kit (PE Biosystem) and analyzed by MALDI-TOF mass spectrometry (Voyager DE Pro, Applied Biosystems). Identification of proteins was performed using MS-Fit software (<http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm>) and verified using tandem MS/MS techniques.

### **<sup>35</sup>S-Methionine labeling of newly synthesized proteins**

HeLa cells were nucleofected with the indicated plasmids and 24 h later were labeled with 150 µCi <sup>35</sup>S-Methionine in 5 ml methionine-free media for the indicated times. Cells were harvested,

counted and equal numbers of cells were lysed. Total protein was precipitated with TCA, and  $^{35}\text{S}$ -CPM was measured for each time point.

### **rRNA [methyl- $^3\text{H}$ ] Methionine and [ $^3\text{H}$ ]Uridine Labeling**

HeLa cells ( $2 \times 10^6$ ) were nucleofected with pcDNA3.1-His, His-tagged NPM or His-NPMdL. Cells were methionine starved for 15 min and labeled with 50  $\mu\text{Ci/ml}$  L-[methyl- $^3\text{H}$ ] methionine (Amersham) for 30 min followed by a 2 h ten-fold excess cold methionine chase. Cells were harvested, counted, and equal numbers were fractionated into cytoplasm and nuclei as described (Yu et al., 2006). Total RNA was isolated from the cytosolic and nuclear fractions with TRIZOL (Invitrogen), and fractionated by gel electrophoresis. *Arf*<sup>-/-</sup> MEFs infected with retroviruses encoding control, His-NPM, or Ras<sup>V12</sup> were labeled with 50  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]Uridine (Amersham) for 45 min followed by a 1 h chase in cold media. Total RNA was isolated from equal numbers of cells ( $1.5 \times 10^6$ ) with TRIZOL (Invitrogen), and fractionated by gel electrophoresis. RNA was transferred to Hybond-NX Membrane (Amersham), cross-linked, sprayed with EN<sup>3</sup>HANCE (Perkin Elmer), and subjected to autoradiography.

### **Ribosome Fractionation**

HeLa cells ( $2 \times 10^6$ ) were nucleofected with pcDNA3.1-His, His-tagged NPM or His-NPMdL. Cells were treated with 50  $\mu\text{g/ml}$  cycloheximide prior to harvesting and counting. Cells ( $3 \times 10^6$ ) were subjected to cytoplasmic or nuclear ribosome fractionation as previously described (Strezoska et al. 2000). For radiolabeling of newly synthesized rRNAs, cells were labeled and fractionated as above. Fractions were collected, RNA extracted with TRIZOL, and  $^3\text{H}$ -CPM was measured for each fraction and graphed for the 40S, 60S, 80S, and polysome peaks.

### **5S rRNA in situ hybridization**

Sections from day E10.5 wild-type and *Npm*<sup>-/-</sup> embryos were mounted on glass slides as previously described (Grisendi et al. 2005). *In situ* RNA FISH analysis was performed using a biotin-labeled probe recognizing the 5S rRNA (GeneDetect.com) according to manufacturer's instructions. Biotin was detected using an in situ hybridization biotin detection kit from DakoCytomation (purple). Nuclear Fast Red (DakoCytomation) counterstaining was used to demarcate nuclei (pink). Whole embryos were visualized at 2X (WT) and 4X (*Npm*<sup>-/-</sup>), respectively and cell images were observed at 60X magnification using a Nikon DIC compound microscope fitted with a Nikon FDX-35 charge-coupled device camera.

### **Densitometry and Image Analysis**

Autoradiograms were scanned using a FlourChem8900 (Alpha Innotech), and densities were determined using NIH ImageJ version 1.33 software.

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**Table 1. Components of the Nucleophosmin Protein Complex <sup>a</sup>**

<b>Protein</b>	<b>Function</b>
rpL4	60S Ribosome subunit
rpL5 <sup>b</sup>	Molecular chaperone for 5S rRNA (Steitz et al., 1998)
rpL13a	60S Ribosome subunit
rpL22	60S Ribosome subunit
rpL27	60S Ribosome subunit
rpS3a	40S Ribosome subunit
rpS5	40S Ribosome subunit
rpS6	40S Ribosome subunit
rpS7	40S Ribosome subunit
Nucleolin <sup>b</sup>	Repress p53 translation (Takagi et al., 2005)
Bop1	Required for rRNA processing (Strezoska et al., 2000)
CPSF6	mRNA processing (Dettwiler et al., 2004)
hnRNP A1	mRNA processing and transport (Dreyfuss et al., 1993)
hnRNP H2	mRNA processing (Dreyfuss et al., 1993)
EF-2	Translational regulation (Carlberg et al., 1990)
Nup50	Nuclear pore complex (Guan et al., 2000)
Nup62	Nuclear pore complex (Radu et al., 1993)
Brca1 <sup>b</sup>	E3 ubiquitin ligase for NPM (Sato et al, 2004)

<sup>a</sup> Isolated by nucleophosmin affinity chromatography and identified by MALDI-TOF or tandem mass spectrometry.  
<sup>b</sup> Known NPM binding proteins

## Figure Legends

### Figure 1. NPM is a potent oncogene in the absence of ARF

**A** *Arf*<sup>-/-</sup> MEFs infected with retroviruses encoding His-tagged NPM or H-Ras<sup>V12</sup> were fixed and subjected to forward and side scatter analysis by flow cytometry. The upper right quadrant represents the cell population with increased size.

**B** *Arf*<sup>-/-</sup> MEFs infected with retroviruses encoding His-tagged NPM or NPMdL were labeled with BrdU for 24 hours prior to fixation and staining with antibodies recognizing BrdU incorporated into replicating DNA. DAPI was used to demarcate cell nuclei. n=3 with the reported standard deviations (\*,  $P>0.01$ , \*\*,  $P>0.005$ ).

**C** *Arf*<sup>-/-</sup> MEFs infected with retroviruses encoding Ras<sup>V12</sup>, His-tagged NPM or NPMdL were seeded ( $3 \times 10^3$  cells) in media containing soft agar and assayed colony formation fourteen days later. Additionally, *Arf*<sup>-/-</sup> MEFs infected with retroviruses encoding His-tagged NPM or NPMdL were seeded in media containing G418 and assayed for foci formation twelve days later.

**D** Primary human breast, prostate, and colon carcinoma tissue microarrays were obtained and stained for NPM protein expression, with representative cancerous tissues staining negative for NPM shown on the left panels, and those staining extremely positive for NPM shown on the right panels. Statistics of NPM protein expression in all three carcinomas are indicated in the insets (% of total slides exhibiting the pattern of staining).

**E** *Arf*<sup>-/-</sup> MEFs infected with retroviruses encoding His-tagged NPM were fixed and stained with antibodies recognizing  $\gamma$ -tubulin. Nuclei were demarcated with DAPI. Centrosomes from over 200 cells were counted for each condition and plotted as a representative experiment (n=3).

**F** *Arf*<sup>-/-</sup> MEFs infected with retroviruses encoding His-tagged NPM were treated with colcemid and fixed. Chromosomes from each condition were prepared and visualized with DAPI.

Expression of His-tagged NPM protein was confirmed by western blot analysis using antibodies recognizing the His moiety.

### **Figure 2. Isolation of endogenous NPM protein complexes**

**A** HeLa cell lysates (600 µg) were injected onto a Sephacryl 300 gel filtration column and fractions were collected as indicated. Proteins were separated by SDS-PAGE and immunoblotted with antibodies recognizing NPM. Gel filtration sizes are indicated above the fractions and the presence of NPM oligomers and monomers is indicated below.

**B** Pooled sizing column fractions (2-16) containing NPM (600 µg) were injected onto an NPM polyclonal antibody affinity column and eluted with an increasing NaCl gradient (0.1-1.0M). Eluted proteins were separated by two-dimensional electrophoresis and visualized with SYPRO-Ruby dye (2D). Spots were excised and digested with trypsin. Tryptic peptides were analyzed by mass spectrometry. Representative MALDI-TOF spectra of labeled spots from above are presented with corresponding identified peptide sequences.

### **Figure 3. NPM nuclear export is required for Protein Synthesis**

**A** HeLa cells were transduced with pcDNA3.1-His (red line), His-NPM (blue line), or His-NPMdL (green line) and incubated for 24 hours. Cells were labeled with <sup>35</sup>S-Methionine for the indicated times. Equal numbers of cells (3x10<sup>6</sup>) were lysed, and TCA-precipitated counts were measured. His-tagged NPM protein expression was measured by anti-His immunoblot to confirm transduction (inset).

**B** HeLa cells were transduced with pcDNA3.1-His (red line), His-NPM (blue line), or His-NPMdL (green line) and incubated for 24 hours. Equal numbers of transduced cells (3x10<sup>6</sup>)

were lysed and separated on sucrose gradients with constant UV (254 nm) monitoring for ribosome and polysome detection. His-tagged NPM protein expression was measured by anti-His immunoblot to confirm transduction (inset).

#### **Figure 4. NPM is the rating-limiting factor for rRNA export**

**A** HeLa cells were transduced with pcDNA3.1-His (CTL), His-NPM, or His-NPMdL. Cells were labeled with [methyl-<sup>3</sup>H] methionine and chased. Equal numbers of labeled cells ( $2 \times 10^6$ ) were subjected to fractionation into cytoplasmic (C) and nuclear (N) extracts. Total RNA extracted from each fraction was separated, transferred to membranes and subjected to autoradiography. Newly synthesized and labeled 28S, 18S and 5.8/5S rRNAs are indicated by arrows. The bottom 5.8S/5S panel is a longer exposure of the same autoradiograph as shown above.

**B** *Arf*<sup>-/-</sup> MEFs infected with retroviruses encoding His-NPM were labeled with [methyl-<sup>3</sup>H] methionine and chased. Equal numbers of labeled cells ( $3 \times 10^6$ ) were subjected to fractionation into cytoplasmic (C) and nuclear (N) extracts. Total RNA extracted from each fraction was separated, transferred to membranes and subjected to autoradiography. Newly synthesized and labeled 28S, 18S and 5.8/5S rRNAs are indicated by arrows.

**C** *Arf*<sup>-/-</sup> MEFs infected with retroviruses encoding His-NPM or Ras<sup>V12</sup> were labeled with [<sup>3</sup>H] uridine and chased. Total RNA extracted from equal numbers of labeled cells ( $3 \times 10^6$ ) was separated, transferred to membranes and subjected to autoradiography. Newly synthesized and labeled 28S, 18S and 5.8/5S rRNAs are indicated by arrows.

**D** HeLa cells were transduced with pcDNA3.1-His (Control) or His-NPM and incubated for 24 hours. Cells were labeled with [methyl-<sup>3</sup>H] methionine and  $3 \times 10^6$  cells were subjected to polysome fractionation. Fractions from the sucrose gradient were isolated and counted for

[methyl- $^3\text{H}$ ] methionine incorporation into newly synthesized rRNAs and assembled cytosolic ribosomes.  $^3\text{H}$ -counts were normalized for the efficiency of label incorporation into total RNA for each condition (\*,  $P>0.005$ ).

**Figure 5. NPM is an essential mediator of ribosome nuclear export**

**A** HeLa cells were nucleofected with scrambled or NPM siRNAs and incubated for 72 hours prior to labeling with [methyl- $^3\text{H}$ ] methionine. Equal numbers of cells ( $2 \times 10^6$ ) were subjected to fractionation into cytoplasmic (C) and nuclear (N) extracts. Total RNA extracted from each fraction was separated, transferred to membranes and subjected to autoradiography. Newly synthesized and labeled 28S, 18S and 5.8/5S rRNAs are indicated by arrows. Western blot analysis using antibodies recognizing NPM was used to visualize efficient knockdown.

**B** HeLa cells were nucleofected with scrambled (solid line) or NPM (dashed line) siRNAs and incubated for 72 hours. Equal numbers of cells ( $3 \times 10^6$ ) were separated on sucrose gradients with constant UV (254 nm) monitoring for ribosome and polysome detection.

**C** Wild-type (WT) and *Npm*<sup>hy/hy</sup> MEFs were labeled with [methyl- $^3\text{H}$ ] methionine and equal numbers of cells ( $3 \times 10^6$ ) were subjected to fractionation into cytoplasmic (C) and nuclear (N) extracts. Total RNA extracted from each fraction was separated, transferred to membranes and subjected to autoradiography. Newly synthesized and labeled 28S, 18S and 5.8/5S rRNAs are indicated by arrows. Lack of NPM protein expression in *Npm*<sup>hy/hy</sup> cells was monitored via western blot analysis using antibodies recognizing NPM.

**Figure 6. Loss of *Npm* prevents 5S rRNA export *in vivo***

*In situ* RNA FISH analysis was performed on wild-type (**A-D**) and *Npm*<sup>-/-</sup> (**E-H**) day E10.5 embryos using a probe recognizing the 5S rRNA (purple). Nuclear Fast Red counterstaining was used to demarcate nuclei (pink). Whole embryos were visualized at 2X (WT) and 4X (*Npm*<sup>-/-</sup>), respectively. Cell images (**A-D** and **E-H**) were observed at 60X magnification.

Figure 1- Maggi

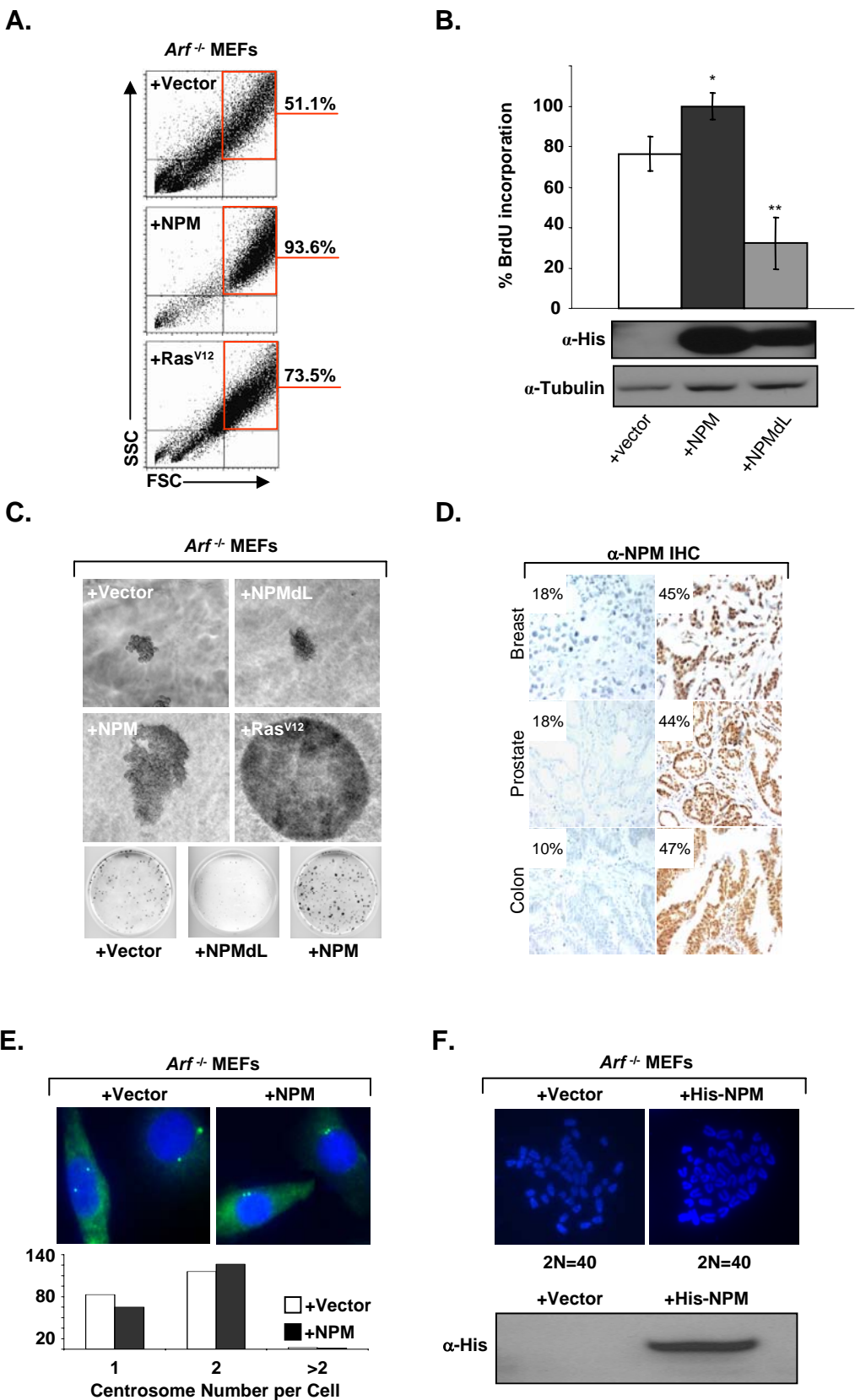


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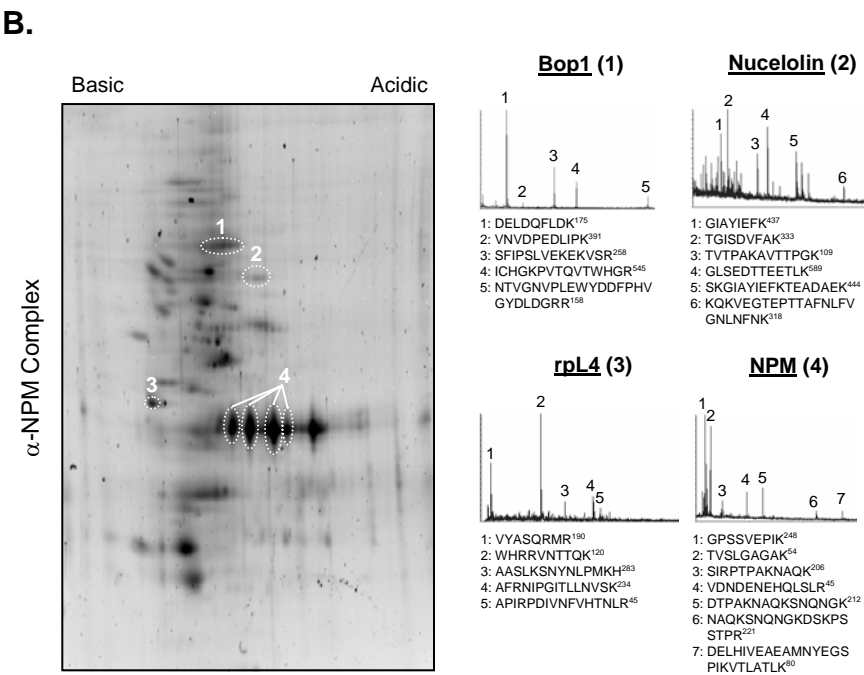
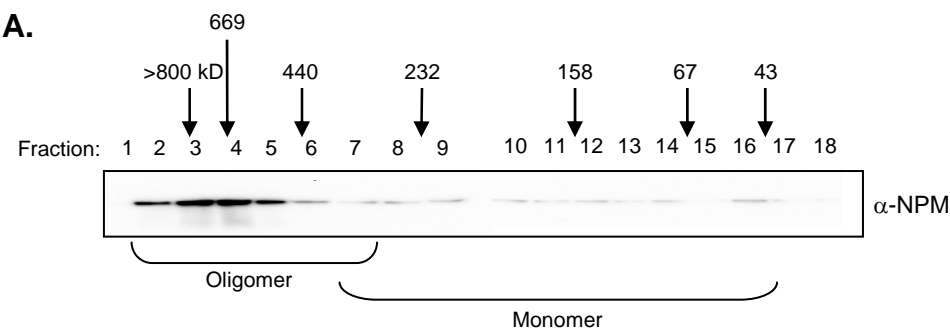
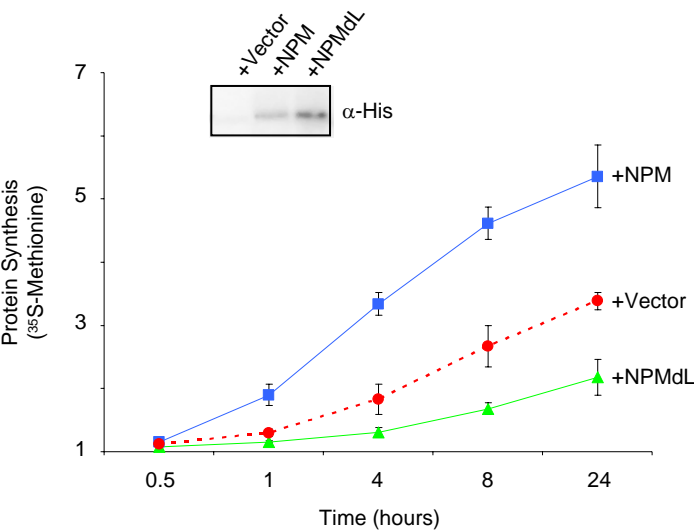




Figure 3- Maggi

A.



B.

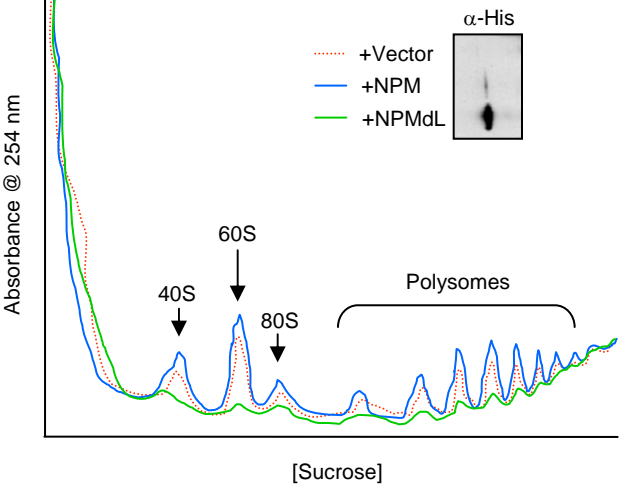


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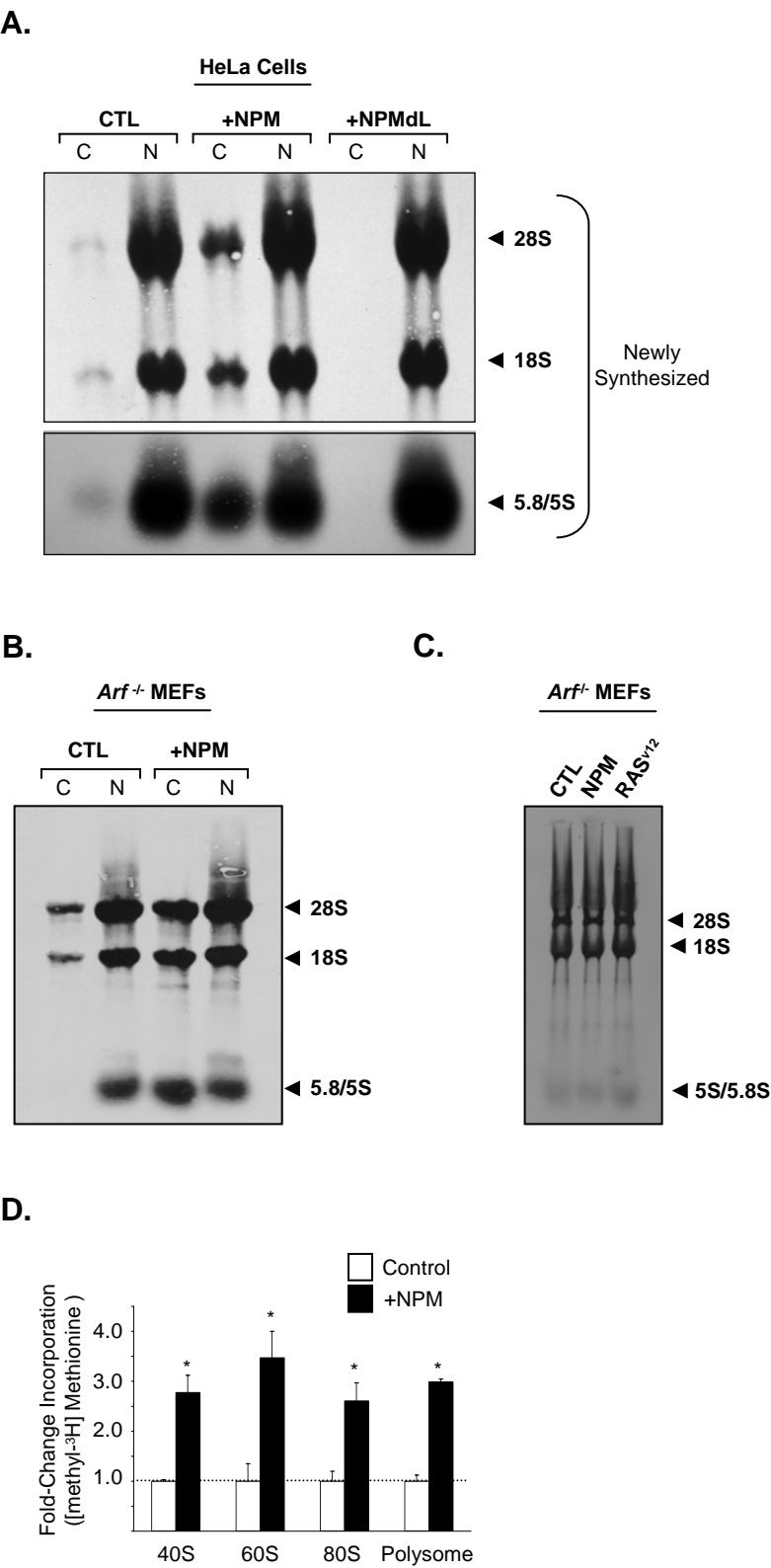


Figure 5- Maggi

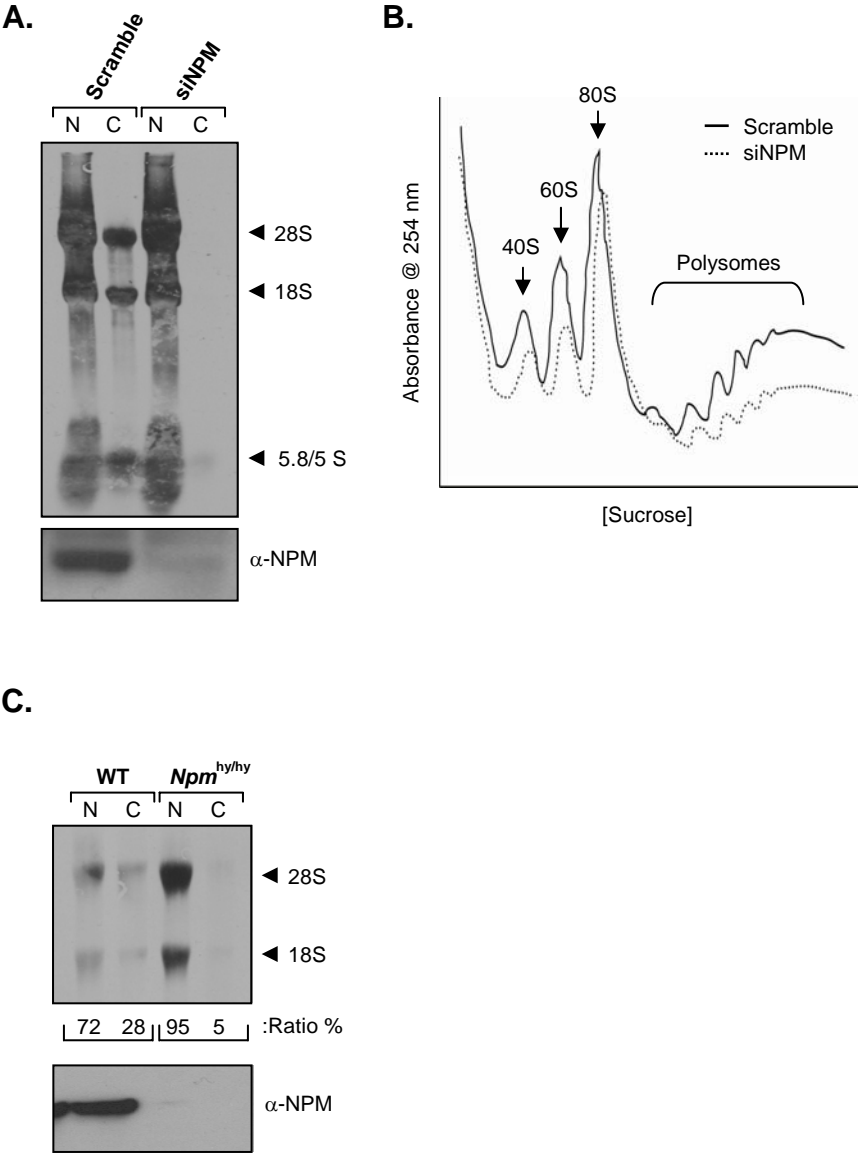
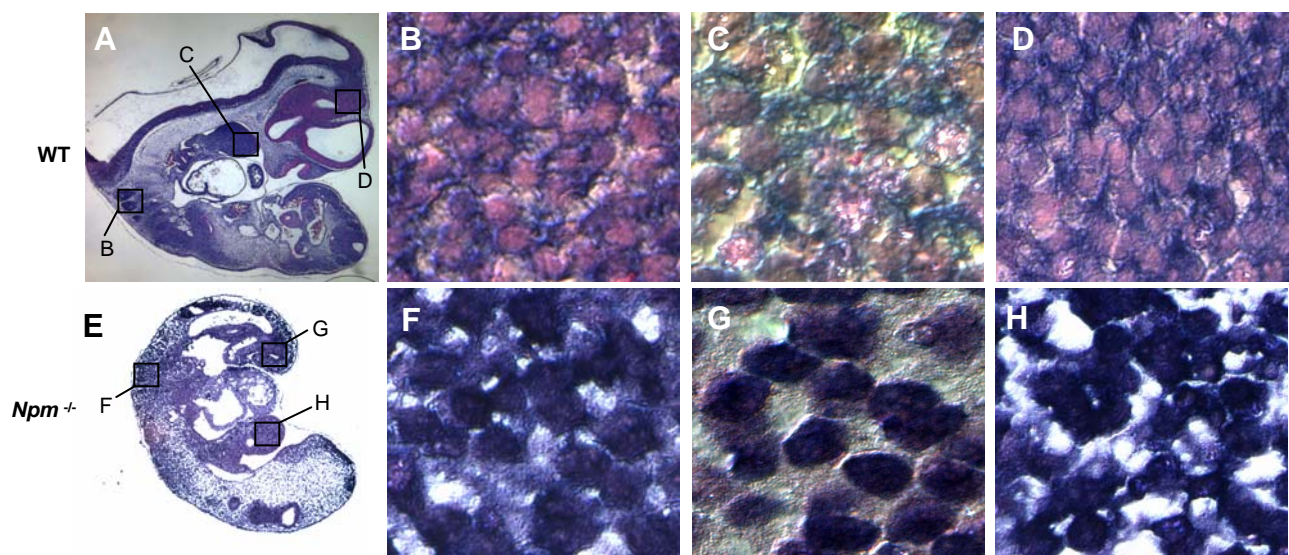
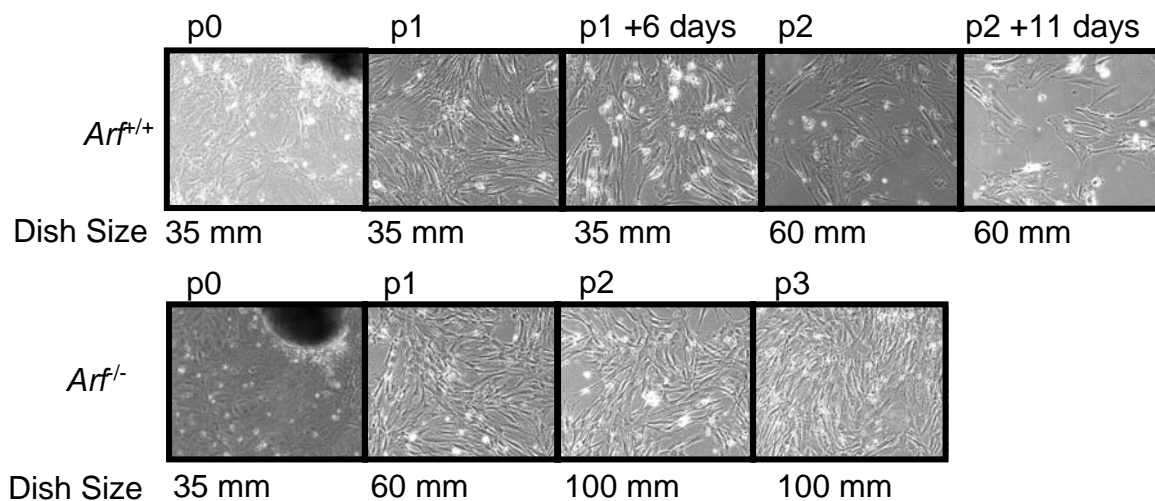
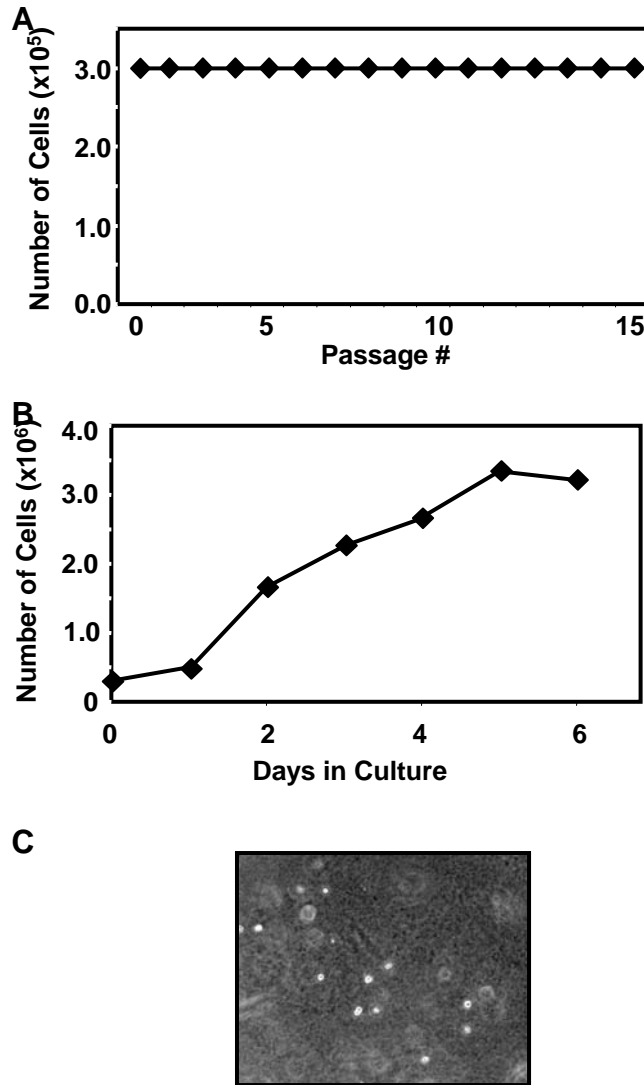


Figure 6- Maggi

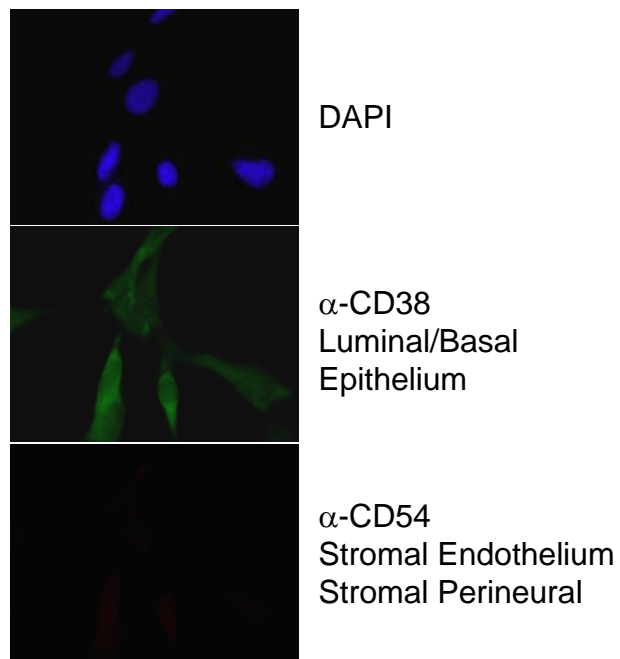




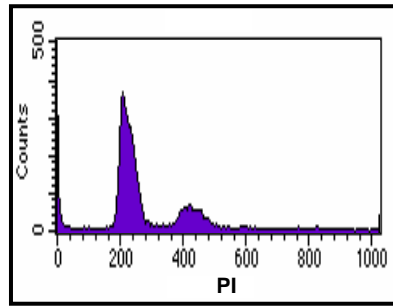
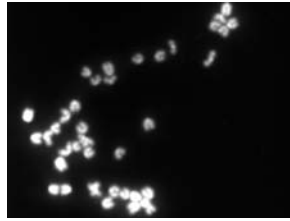
**Figure 1. *Arf*<sup>-/-</sup> Prostate Cell Cultures.** Prostates isolated from *Arf*<sup>+/+</sup> and *Arf*<sup>-/-</sup> mice were chopped in to ~1mm<sup>3</sup> pieces and grown in 35mm wells of a 6 well plate. When confluent the cells were passaged to larger plates as indicated. These data are representative of 2 independent isolations.



**Figure 2. *Arf*<sup>-/-</sup> Prostate Cells are Immortal not Transformed.** **A** *Arf*<sup>-/-</sup> prostate ( $3 \times 10^5$  cells) were seeded on 35 mm dishes and  $3 \times 10^5$  cells were passaged every 3 days. **B** *Arf*<sup>-/-</sup> prostate cells ( $3 \times 10^5$  cells) were seeded on 100 mm dishes and cell number was determined every 24 h. **C** *Arf*<sup>-/-</sup> prostate cells (1000 cells) were seeded in media containing soft agar and colony formation was assessed fourteen days later. 3T3 protocol (**A**) and growth curve (**B**) are representative of 2 independent experiments performed in triplicate. Colony formation in soft agar (**C**) is representative of 3 independent experiments performed in quadruplicate.

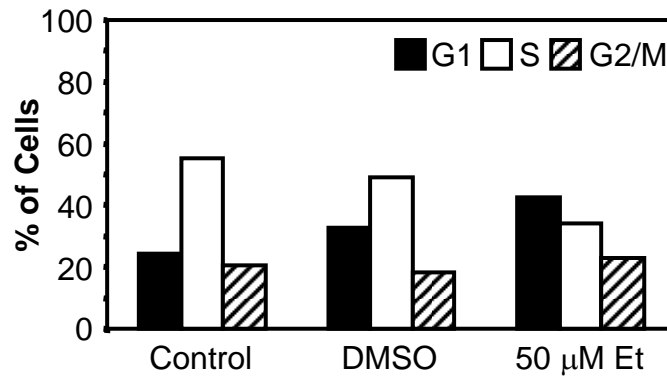
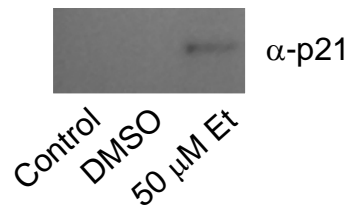


**Figure 3. *Arf*<sup>-/-</sup> Prostate Cells are Epithelial Cells.** *Arf*<sup>-/-</sup> prostate cells were stained with antibodies against CD38 (Epithelial Prostate marker) or CD54 (Stromal Prostate marker). Nuclei are demarcated with DAPI. These data are representative of two independent isolations.

**A****B** $2N=40$ 

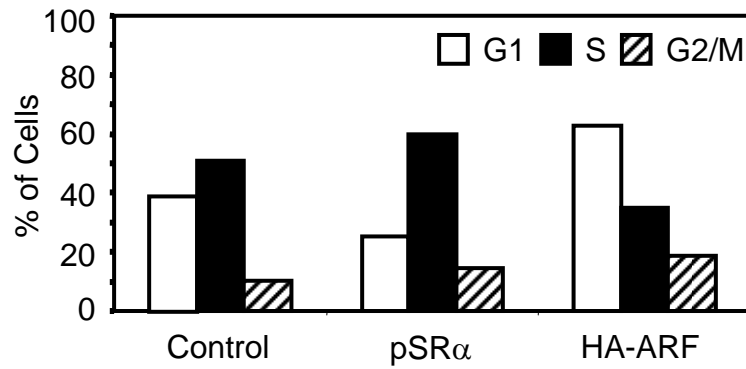
**Figure 4. *Arf*<sup>-/-</sup> PEpC are Diploid.** **A** *Arf*<sup>-/-</sup> PEpC were fixed and stained with PI prior to DNA content analysis by flow cytometry. **B** *Arf*<sup>-/-</sup> PEpC were treated with colcemid and fixed. Chromosomes were prepared and visualized with DAPI. Results from flow cytometry (**A**) are representative of 3 independent experiments. Results from Chromosome analysis (**B**) are representative of 5 metaphase spreads.



**A****B**

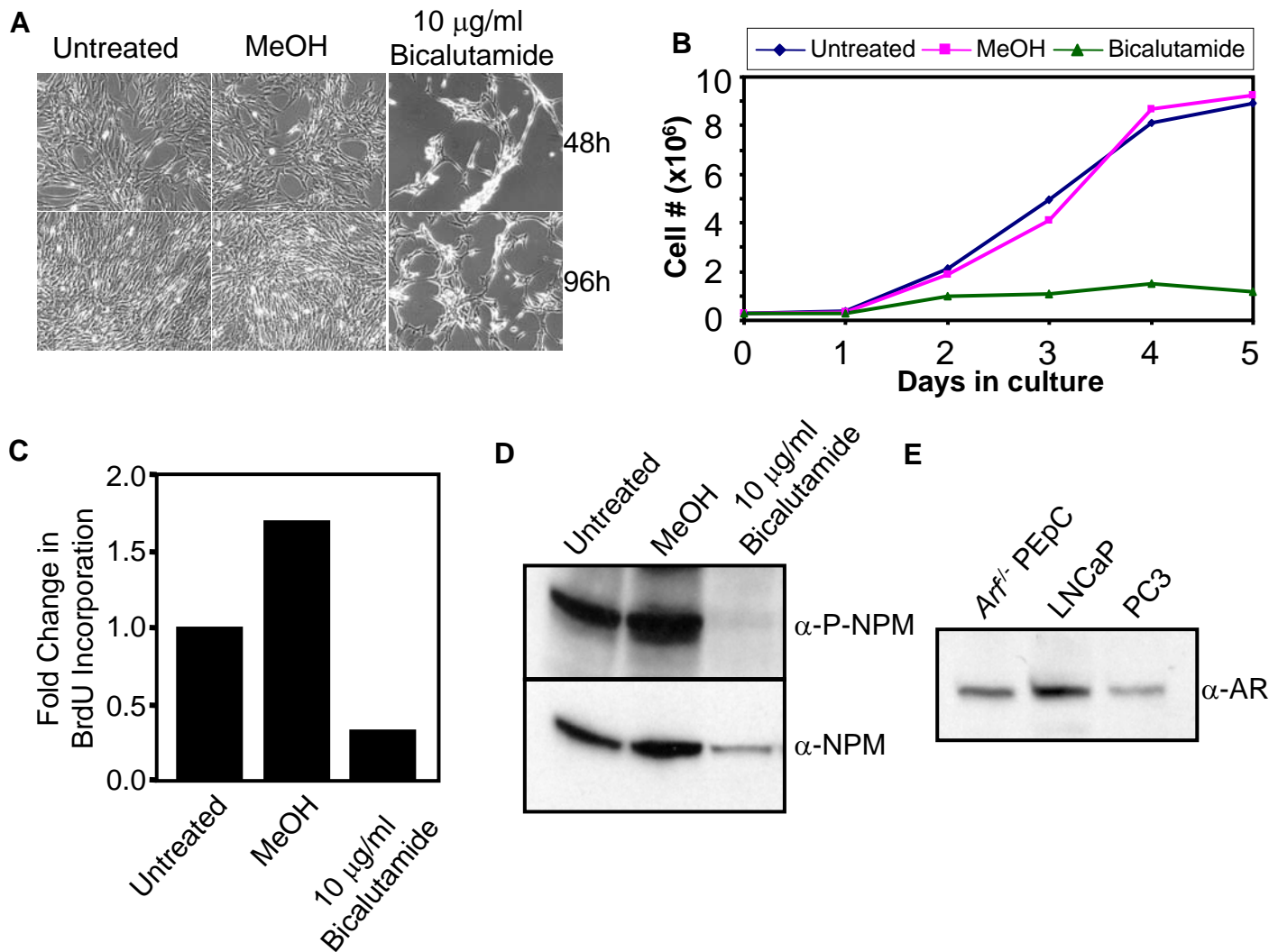
**Figure 5. *Arf*<sup>-/-</sup> PEpC Have an Intact p53 Response.** **A** *Arf*<sup>-/-</sup> PEpC ( $1 \times 10^6$ ) were treated for 18 h with DMSO or 50  $\mu$ M Etoposide prior to being fixed and stained with PI. DNA content was analyzed by flow cytometry. **B** A portion of the cells from **A** were used in western blot analysis to assess p21 levels. These data are representative of 3 independent experiments.

**Figure 6. p19ARF Overexpression Inhibits *Arf*<sup>-/-</sup> PEpC Cell Cycle Progression**

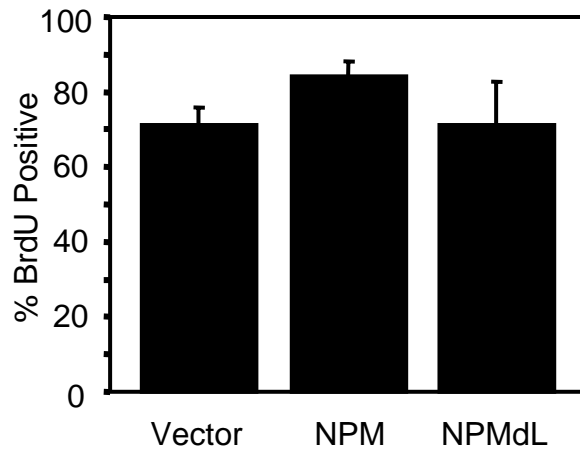


**Figure 6. p19ARF Overexpression Inhibits *Arf*<sup>-/-</sup> PEpC Cell Cycle Progression.** *Arf*<sup>-/-</sup> PEpC were infected with HA-p19ARF expressing retroviruses. DNA content was analyzed 48 h later by flow cytometry. This data is representative of 2 independent experiments.

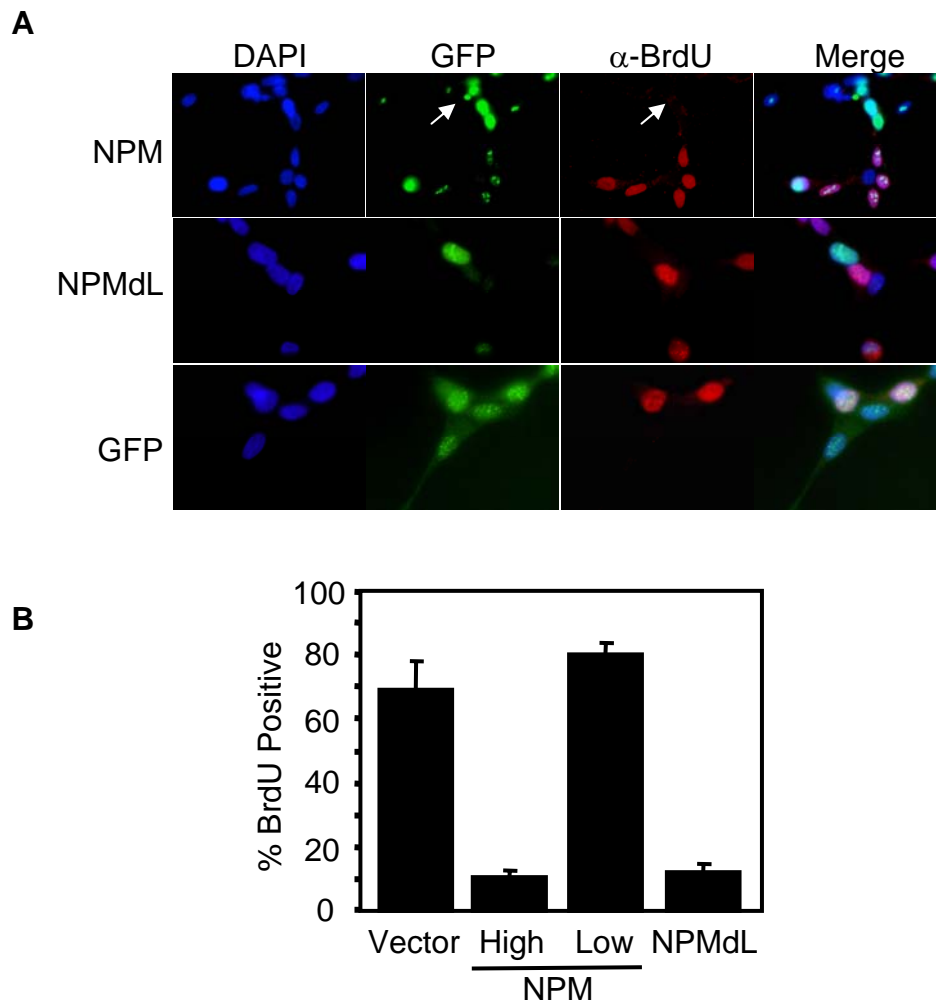
**Figure 7. Effects of Androgen Receptor Inhibition on *Arf*<sup>-/-</sup> PEpC Cell Growth**



**Figure 7. Androgen Receptor Signaling is Required for *Arf*<sup>-/-</sup> PEpC Growth.** **A** *Arf*<sup>-/-</sup> PEpC (1x10<sup>6</sup>) were treated with MeOH or 10 µg/ml bicalutamide as indicated and cell density assessed at the indicated times. **B** *Arf*<sup>-/-</sup> PEpC (3x10<sup>5</sup>) were seeded onto 100 mm dishes in the presence or absence of MeOH or 10 µg/ml bicalutamide as indicated and cell number determined every 24 h. **C** *Arf*<sup>-/-</sup> PEpC (7.5x10<sup>4</sup>) were seeded onto glass cover slips in the presence or absence of MeOH or 10 µg/ml bicalutamide for 48 h. Cells were labeled with 10 µM BrdU for 18 h prior to fixation and staining with antibodies recognizing BrdU incorporated into replicating DNA. DAPI was used to demarcate cell nuclei. **D** *Arf*<sup>-/-</sup> PEpC treated with MeOH or 10 µg/ml bicalutamide for 96 h were analyzed by western blot with antibodies against phosphorylated Thr198 NPM or NPM as indicated. **E** Cell lysates (10 µg) from *Arf*<sup>-/-</sup> PEpC, LNCaP, and PC3 cells were analyzed for androgen receptor expression by western blot. These data are representative of three independent experiments.

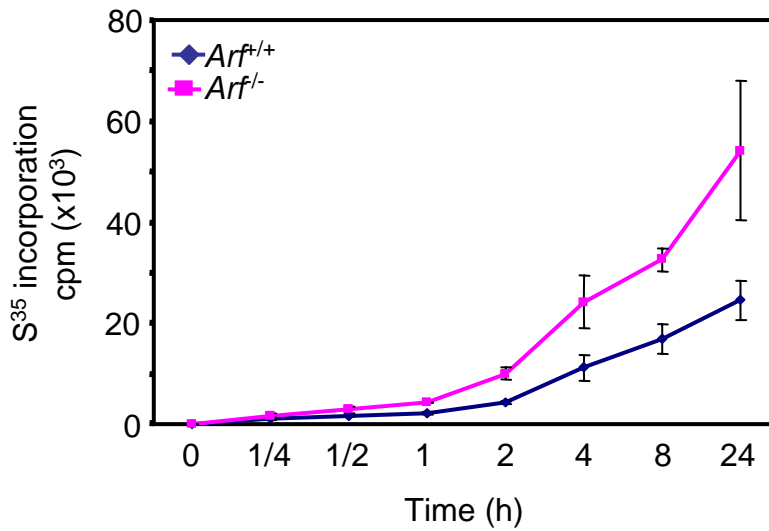


**Figure 8. PC3 Cell S Phase Progression Is Not Affected By NPM Shuttling.** PC3 cells nucleofected with empty vector or GFP-tagged NPM or NPMdL were labeled with BrdU for 18 h prior to fixation and staining with antibodies against BrdU incorporated in to replicating DNA. DAPI was used to demarcate nuclei. These data are representative three independent experiments performed in triplicate  $\pm$  SEM.

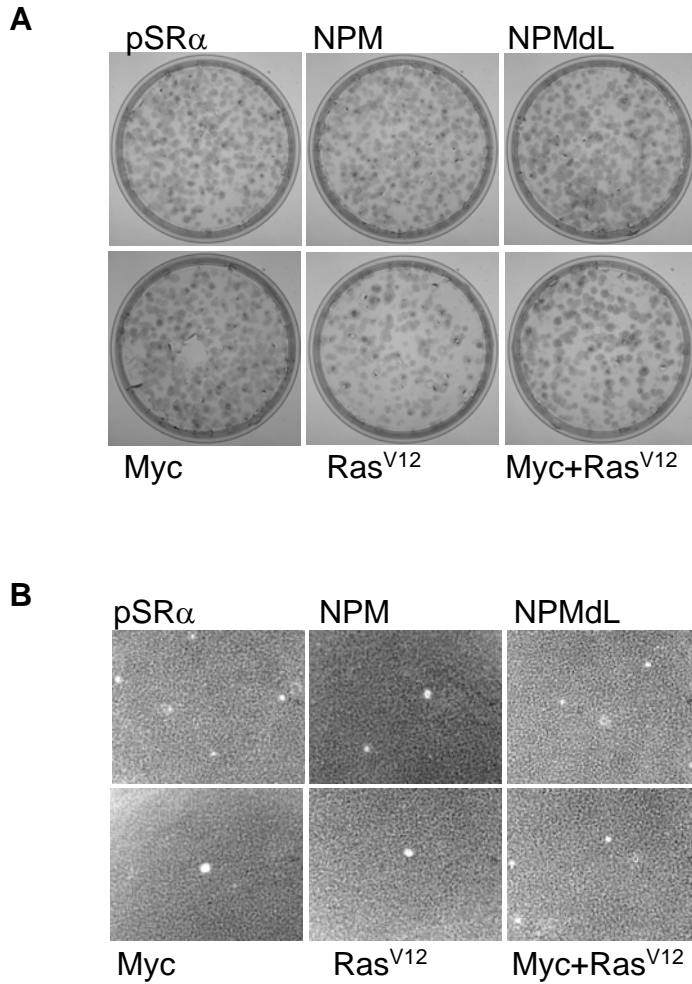


**Figure 9. LNCaP Cell S Phase Progression Is Affected By Inhibition of NPM Shuttling or High Expression of NPM.** **A** LNCaP cells nucleofected with empty vector or GFP-tagged NPM or NPMdL were labeled with BrdU for 18 h prior to fixation and staining with antibodies against BrdU incorporated in to replicating DNA. DAPI was used to demarcate nuclei. **B** Quantification of BrdU positive cells shown in A. These data are representative two independent experiments performed in triplicate  $\pm$  SD.

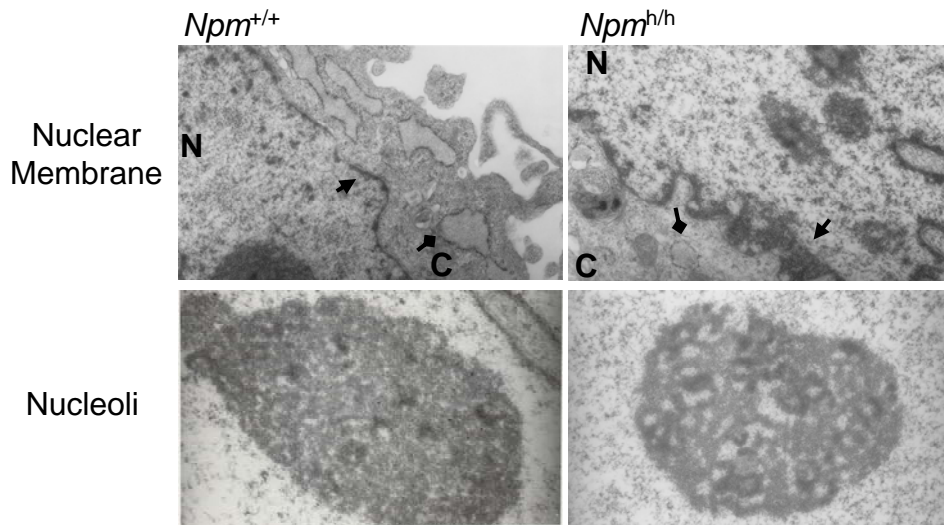
**Figure 11. *Arf* Loss Increases Protein Synthesis Rates of PEpC**



**Figure 10. *Arf* loss Increases Protein Synthesis Rates of PEpC.** *Arf*<sup>+/+</sup> or *Arf*<sup>-/-</sup> PEpC were labeled with <sup>35</sup>S-methionine in methionine-free media for the indicated times. Equal numbers of cells (2.5×10<sup>4</sup>) were lysed and TCA-precipitable counts were measured. This data is representative of two experiments performed in triplicate ± SD.



**Figure 11. *Arf*<sup>-/-</sup> PEpC Cannot Be Transformed.** *Arf*<sup>-/-</sup> PEpC were infected with the indicated retroviruses. **A** Forty-eight hours post infection  $3 \times 10^5$  cells were seeded in media containing 800  $\mu\text{g/ml}$  G418 and foci formation was assessed fourteen days later. **B** Forty-eight hours post infection  $1 \times 10^4$  cells were seeded in media containing soft agar and 800  $\mu\text{g/ml}$  G418. Colony formation was assessed fourteen days later. These data are representative of two independent experiments.



**Figure 12. Loss of *Npm* Inhibits Ribosome Nuclear Export and Alters Nucleolar Morphology.** *Npm*<sup>+/+</sup> or *Npm*<sup>h/h</sup> Mouse Embryo Fibroblasts were fixed and subjected to electron microscopic analysis. **Top panels** Arrows → point to nuclear membrane with increased ribosomes in the *Npm*<sup>h/h</sup> cells. Diamond headed arrows —◆ point to cytoplasmic rough ER which is dramatically decreased in *Npm*<sup>h/h</sup> cells. **Bottom panels** Increased magnification focused on nucleoli of *Npm*<sup>+/+</sup> or *Npm*<sup>h/h</sup> cells.